

**STRUCTURAL CHANGES IN THE RABBIT PENILE
ARCHITECTURE IN INDUCED HYPOGONADISM**

By

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LIST OF ABBREVIATIONS

ADT – Androgen deprivation therapy

DHT – Dihydrotestosterone

ED – Erectile dysfunction

GnRH – Gonadotropic hormone-releasing hormone

ICP – Intracavernosal pressure

MPW – Masculinization programming window

SHBG – Sex hormone binding globulin

SPSS – Statistical Product and Service Solutions

TGF – Transforming growth factor

VEGF – Vascular endothelial growth factor

SUMMARY

Background: The penile body consists of erectile masses that contain vascular sinusoids lined by endothelial cells and trabeculae which consist of smooth muscles and connective tissue fibers. The integrity of the trabeculae and vascular sinusoids is vital in the physiology of penile erection and so their structural alterations may result in erectile dysfunction. Androgenic hormones may be important in maintaining this structural integrity. Accordingly, decline in androgen levels in normal aging, androgen deprivation therapy and disorders that either damage the testes or reduce gonadotropin stimulation are known to cause hypogonadism associated with erectile dysfunction. However, the direct link between hypogonadism and erectile dysfunction is relatively underexplored and the anatomical basis is altogether undescribed. Understanding of the structural basis of erectile dysfunction following hypogonadism may inform new frontiers of investigation and patient follow-up, in addition to giving more evidence for hormonal therapy. Alterations may also be important when analyzing tissues from penile biopsy in various gonadal states, and in tissue engineering for phallic grafting.

Study Objective: To describe possible structural changes in the penile smooth muscle, connective tissue morphology and penile vascular system that may occur following gonadal androgen hormone deprivation after bilateral orchiectomy.

Materials and Methods: Experimental animals were obtained from a rabbit farm. Fifteen adult male rabbits were used for the study. Nine of these were castrated to induce hypogonadism (intervention group) and six were not (non-intervention group). Surgical castration was done under local anesthesia at the beginning of the study in the intervention group using the prescrotal approach. Five rabbits (3 from intervention and 2 from the non-intervention groups) were perfused

three weekly from the time of castration. Penile lengths were measured using a digital Vernier caliper (accuracy 0.5mm) at the beginning for each animal, and every three weeks until the specific rabbit was perfused. After perfusion, tissues were fixed for a period of 24 hours in 10% formal saline then processed for light microscopy. Masson's trichrome stain was used to display the smooth muscle and collagen fiber profiles, while Weigert's elastin stain with Van Giesson counterstain was used to demonstrate elastic fibers. Stereological techniques were used in morphometric analysis of functional components – vascular volume in corpus cavernosum and corpus spongiosum, smooth muscle density and connective tissue fiber components. Smooth muscle and connective tissue contents were determined using the Cavalieri principle of point counting and data expressed as volume densities (%). The ratios of smooth muscle and connective tissue components were estimated from the densities of each component. The Student's t test was applied for mean comparisons, with P-value < 0.05 considered significant. Thickness of smooth muscle cells and connective tissue fibers was done by point sampled intercepts. Data was coded and analyzed using SPSS Version 17.0 and presented in tables.

Results: An average reduction in the non-erect penile length by 0.7%, 3.4% and 8.7% in the castrated group at the end of the third, sixth and ninth week respectively was noted. The volumetric density of the trabecular smooth muscle cells of the erectile bodies decreased from a normal of about 64% to about 12% at the end of nine weeks after castration. The proportion of collagenous fibers to smooth muscles in the trabecular increased significantly, implying erectile tissue fibrosis with a longer exposure to hypogonadal state. Spongiosal fibrosis was more marked than cavernosal fibrosis. The lamella arrangement of collagen fibers in the inner layer of the tunica albuginea was interrupted in the hypogonadic rabbits, and the inter-cavernosal septum disintegrated in the intervention group but preserved in the non-intervention group. The amount of elastic fibers in the

cavernosal trabecular system decreased with duration of hypogonadism. There was cavernosal artery fibrosis, vascular leakage and general narrowing or collapse of subtunical vessels in the castrated group while the integrity of these vasculature was preserved in the non-castrated rabbits. There was progressive fat cell accumulation in the subtunical zones of the castrated rabbits, especially after six and nine weeks of hypogonadism. Also observed was intratunical adipocyte accumulation after nine weeks of hypogonadic state. The severity of the changes was proportional to the duration after castration.

Conclusion: Castration induces diminutive changes in all tissue components of the penile structure. These anatomical changes may underpin erectile dysfunction in hypogonadism. Androgen therapy is recommended in hypogonadal states to reserve normal penile physiology.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The penis has a root, a body and a glans. The root consists of two crura and a bulb located in the urogenital triangle. These are firmly attached to the pubic arch and perineal membrane, and are covered by ischiocavernosus and bulbospongiosus muscles respectively (Hsu et al., 2004). The body is a tubular appendage with three distinct cylindrical compartments, each one of which is encased by a thick connective tissue capsule, the tunica albuginea. The ventral compartment, which also contains the penile urethra, is the corpus spongiosum while the paired dorsal compartments are the corpora cavernosa. These three constitute the penile erectile masses, which are vast spongelike systems of irregular vascular spaces fed by afferent arteries and drained by efferent veins (Boom and Fawcett, 1968). These tissues therefore act as vascular capacitance organs (Saenz de Tejada et al., 1991). The corpora cavernosa share a common septum in the pendulous portion of the penis with many perforations that allow free passage of blood from one side to the other, allowing the two corpora to function essentially as a single unit. These corpora cavernosa end blindly under cover of the glans penis, which is a distal enlargement of the corpus spongiosum (Hsu et al., 2004).

Penile erectile tissues are made up of numerous vascular sinusoids lined by endothelial cells and surrounded by a rich trabeculae network which consist of smooth muscles and connective tissue fibers (Goldstein and Padma-Nathan, 1990; Saenz de Tejada et al., 1991; Pinheiro et al., 2000; Babinski et al., 2005; Mirone et al., 2009). These structures are believed to play a key role in the penile erection. For example, the smooth muscle and connective tissue are important in

maintaining the penile vascular tone, hence determining the efficiency of erection (Traish, 2009; Mirone et al., 2009). Since these components are vital in the physiology of penile tumescence and detumescence, alterations in their quantity and organization may lead to functional impairment hence erectile dysfunction (Shafika et al., 2010). Androgenic hormones may be important in maintaining the structural integrity of the penis since their deficiency is coupled with increased incidence of erectile dysfunction. The direct link between androgenic hormone deficiency and erectile dysfunction is, however, relatively underexplored.

Previous studies have shown that mesenchymal stem cells may transform into adipose lineage instead of smooth muscle in androgen deficiency (Bhasin et al., 2003; Singh et al., 2003; Traish et al., 2005). If this occurs in the penis, it may have significant functional prejudice to this organ. There are conflicting reports on the association between androgen deficiency and penile connective tissues, but certainly there are alteration in the penile fibroelastic properties (Shen et al., 2003). The organization and proportions of collagen and elastic fibre components, however, remain undescribed. The association between the penile erectile tissue vascular spaces and androgen level are also hitherto undescribed.

The current study therefore sought to describe possible structural changes in the penile length, penile smooth muscle cells, connective tissue (collagen and elastic) fiber morphology and penile erectile tissue vascular spaces that may occur following gonadal androgen hormone deprivation after bilateral orchiectomy. Understanding the penile architectural changes in hypogonadism would help in interpretation of penile biopsy specimens. It may also inform new frontiers of investigation and patient follow-up in hypogonadic conditions, in addition to giving more evidence for the importance of hormonal therapy.

1.2 Literature Review

The penis evolved as an internal fertilization device, consisting of root, body and glans (Gallup et al., 2003; Gallup, 2004). The penile body consists of specialized vascular beds characterized by a complex trabecular angioarchitecture composed of smooth muscle cells, endothelial cells, neuronal cells, and fibroblasts, interacting with collagen and elastic fibers (Boom and Fawcett, 1968; Goldstein and Padma-Nathan, 1990; Saenz de Tejada et al., 1991; Pinheiro et al., 2000; Babinski et al., 2005; Mirone et al., 2009). These structures, believed to play a key role in the penile erection, may be altered in androgen hormone deficiency.

1.2.1 Types of Penis

There are two types of penis: the musculocavernous and fibroelastic penis (Rowen et al., 2009). The musculocavernous type contains a lot of erectile tissue and little connective tissue so during erection there is both an increase in length and diameter of the penis as seen in stallion, primates and cat. It contains large vascular spaces divided by thin septa, hence relatively larger volume of blood is required to achieve penile erection. The fibroelastic type of penis has a sigmoid flexure, and contains large amounts of connective tissue. Erection of this type of penis largely occurs by straightening of the sigmoid flexure, causing increase in length as seen in ruminants and swine. It requires little blood to achieve full erection and there is no significant increase in diameter. Some animals like dogs and cats, however, have the distal end of the cavernosum ossified to become the os penis.

1.2.2 Relevant Surgical Anatomy of the Male Rabbit

The urogenital anatomy of male rabbit is unique among placental mammal species, and common in marsupial species (Donnelly, 2004; Capello, 2005). The penis is located caudal to the testicles which lie in two separate hemiscrotal sacs. The other important anatomical peculiarity is the

existence of an open inguinal canal, making rabbits (and rodents) “functional cryptorchids.” The testicles of rabbits are elongated and not round, and the epididymis is clearly visible at the caudal pole of the testicle. There is also significantly less peri-testicular fat, and the glans of the penis is not well developed, is tapered, and covered by a prepuce (Donnelly, 2004). These anatomical peculiarities of male rabbits have important implications with regard to orchiectomy. Ligation of the open inguinal canals is recommended during the surgical procedure in order to prevent hemiscrotal herniation of abdominal viscera such as intestinal loops or the urinary bladder. The position of the penis caudal to testicles makes a prescrotal approach with a single incision on the midline possible.

1.2.3 Mechanisms of Penile Erection

Erection of the penis occurs when more blood enters the organ by way of arterial supply than leaves by veins (Boom and Fawcett, 1968). This enlarges the penis and makes it turgid. In the musculocavernous type of penis, enlargement occurs in all directions. Penile erection is a hemodynamic response to a combination of vasodilatory signaling mechanisms coupled with reduced vasoconstrictor activity, both from central and peripheral centers. The balance between contractant and relaxant factors control the degree of contraction of the smooth muscle of the corpora cavernosa and determines the functional state of the penis (Andersson, 2011). A normal erection therefore requires healthy penile fibrovascular tissues and an intact neuroendocrine signaling (Traish and Guay, 2006). In flaccid state the sympathetic neuronal input is dominant, releasing neurotransmitters such as noradrenaline that maintain cavernosal smooth muscle contraction. This causes vasoconstriction and diminished blood flow to the penis. During

erection however, the penis acts as a capacitor that accumulates blood under systolic pressure (Saenz de Tejada et al., 1991; Traish, 2009).

1.2.4 Androgens and Penile Structure

Androgens are the male sex hormones responsible for embryonic development of the male reproductive organs. In the penis, androgen action predetermines penile length (Welsh et al., 2010), regulates differentiation of precursor cells into trabecular smooth muscle (Traish, 2009), and regulate formation of the urethral orifice and internal prepuce (Blaschko et al., 2013). Testosterone is the main androgen but other circulating androgens also exist; these are dihydrotestosterone, dehydroepiandrosterone, androstenedione and androstenediol. Testosterone is converted to dihydrotestosterone through the action of 5-alpha reductase enzyme. Androgen receptors are widely distributed in several tissues. Through classic cytosolic androgen receptors or membrane receptors, testosterone induces genomic and non-genomic effects, respectively (Lopes et al., 2012). Endothelial cells and smooth-muscle cells are the main cellular targets for direct androgen effects in penile tissues (Mirone et al., 2009).

A number of studies have demonstrated the importance of androgens in normal penile erection (Foresta et al., 2004; Morales et al., 2004; Montorsi and Oettel, 2005; Traish and Guay, 2006; Saad et al., 2007). Experimental evidence indicates that testosterone modulates the synthesis and bioavailability of nitric oxide and, consequently, endothelial function, which is key for a healthy vasculature (Lopes et al., 2012). Moreover, androgens promote endothelial cell survival, reduce endothelial expression of pro-inflammatory markers, and inhibit proliferation and intimal migration of vascular smooth-muscle cells (Foresta et al., 2008). The regulatory effect of

androgens on growth and differentiation of smooth muscle cells has been shown where androgens induce pluripotent stem cells along a vascular smooth muscle lineage and inhibit the differentiation of the same cells into an adipocyte lineage (Bhasin et al., 2003; Singh et al., 2003).

With age, healthy men experience a physiological but important decline in the plasma concentrations of bioactive androgens, although not as abrupt as described for the estrogen levels in women around menopause (Valenti, 2005; Nardoza et al., 2011; McGill et al., 2012). This age-related decline in androgens is caused by different simultaneous mechanisms, such as primary structural gonadal impairment, age-related degenerative modifications of the pituitary gland, deficits of the neurohypothalamic system, where the aging hypothalamus tends to pulse more slowly with less gonadotropic hormone-releasing hormone (GnRH) production (Alexandersen and Christiansen, 2004; Valenti, 2005). Together, these factors define the so called "idiopathic pathway". Generally, the decline in androgen levels is exacerbated by many other age-associated clinical conditions that can interfere with testosterone metabolism: these represent the "secondary pathway" (Valenti, 2005). The secondary pathway includes metabolic abnormalities such as the age-associated increase in the concentration of serum sex hormone binding globulin (SHBG), with a consequent decrease in free testosterone (Valenti, 2005). Genetic factors (Meikle et al., 1986), chronic diseases (Turner and Wass, 1997; Miner et al., 2011), chronic medications (MacAdams et al., 1986), obesity (Vermeulen et al., 1996; Anderson et al., 2012) and lifestyle factors (Travison et al., 2007) have also been implicated.

The prevalence of age-related androgen deficiency may be as high as 30% in men aged 40–79 years (Allan and McLachlan, 2004; Liu et al., 2009), and in up to 12% the hypotestosteronemia can be associated with clinical symptoms (Liu et al., 2009). Apart from aging, androgen levels

may also be reduced in other conditions such as androgen deprivation therapy for patients with prostate cancers (Basaria et al., 2002) and disorders that either damage the testes or reduce gonadotropin stimulation (Eunice et al., 2007).

Male hypogonadism causes a wide spectrum of abnormalities depending on the timing of onset. During embryonic development, this causes various congenital anomalies of the internal and external genitalia (Foster and Harris, 2005). Some of these are as shown in table 1.

Table 1: Effects of Androgen Deficiency According to Time of Onset

Time at onset	Effects of Androgen Deficiency
1 st Trimester	Incomplete virilization of external genitalia hence ambiguous genitalia
	Incomplete development of the Wolfian Ducts to form the male internal genitalia
3 rd Trimester	Micropenes (Bin-Abbas et al., 1999; Traish and Guay, 2006)
Puberty	Incomplete pubertal maturation (Eunice et al., 2007)
	Eunuchoidal body habitus (Eunice et al., 2007)
	Poor muscle development and reduced peak bone mass (Araujo et al., 2007)
Post-puberty	Decrease in energy, mood and libido (Morales et al., 2004; Araujo et al., 2007; Gurbuz et al., 2008)
	Decrease in sexual hair, hematocrit, muscle mass and strength, and bone mineral density (Araujo et al., 2007; Jankowska et al., 2009)
Adulthood and Senescence	Decrease libido, erectile dysfunction, infertility, fatigue, decreased muscle strength, increased adiposity, irritability, depressed mood, small testis and reduced beard (Eunice et al., 2007).

Low androgen levels are also associated with erectile dysfunctions. This may be through multiple mechanisms such as alteration in the synthesis and release of neurotransmitters, altered smooth muscle responsiveness to neurotransmitters, or change of fibroblastic properties of the erectile tissues (Traish et al., 2003). At molecular level, hypogonadism may impair several mechanisms, leading to pathologic structural remodeling (Mirone et al., 2009). This includes apoptosis of endothelial and smooth-muscle cells (Liu et al., 2007). Moreover, low androgen levels impair proliferation, migration, and homing of endothelial progenitor cells as well as myogenic

differentiation of mesenchymal progenitor cells. It is also associated with increased risk for atherosclerotic vascular wall remodeling (Demirbag et al., 2005).

Despite this elaborate knowledge on the role of androgens in development of the male reproductive system and secondary male characteristics, little is known on their role in maintaining the integrity of the penile architecture postnatally.

1.2.5 Penile Length

The penile length varies between different animals. The average penile length in adult men is about 9.0-9.5 cm when flaccid, 14.5-15.0 cm when stretched in the flaccid state and 12.8-14.5 cm when maximally erect (Mondaini et al., 2002; Dillon et al., 2008). There are hardly any published reports of normal rabbit penile lengths. Variability however arises between standardization of penile measurements. Although there is no standard technique for measuring penile size, there appears to be consensus among researchers that penile length should be measured on the dorsum of the penis beginning from the pubopenile junction (base of the penis) to the tip of the glans as the most distant point (Wessells et al., 1996; Dillon et al., 2008).

Evaluation of penile size is a routine clinical procedure in the diagnosis and prognosis of patients who are candidates for reconstructive surgery of the penis (Chen et al., 2000). Although penile shortening has been documented, there is no reliable data to support or refute the overall effect of this on male sexuality. Subnormal penile sizes have been reported in humans suffering from androgen insensitivity (Hlaskova et al., 2009), in postprostatectomy patients (Haliloglu et al., 2007; McCullough, 2008; Yu et al., 2010; Park et al., 2011; Vasconcelos et al., 2012) and patients undergoing radiotherapy (Hall et al., 1995; Haliloglu et al., 2007; Parekh et al., 2013). Androgenic hormone deficiency occurring during embryonic development is also a known cause of micropenes

(Bin-Abbas et al., 1999; Traish and Guay, 2006). The maximal growth potential of the penis is in fact predetermined during embryonic period when the level of androgenic activity is high. However, a normal postnatal androgenic action is vital for the penis to achieve this size (Welsh et al., 2008; Macleod et al., 2010). Our current question is whether the normal penis would actually decrease in size, and if so by what proportion, in conditions of androgen deprivation.

1.2.6 Penile Smooth Muscle Cells

The penile smooth muscle cells are disposed in clusters within the trabeculae of the cavernous spaces. They are oriented in all directions and establish at least 2 insertions upon elements of the fibrous skeleton of the erectile tissues (Goldstein and Padma –Nathan, 1990). Similar to vascular smooth muscle cells, these cells interact with endothelial cells hence helping in regulating blood flow within cavernous spaces (Mirone et al., 2009). In fact, the intracavernosal smooth muscle tone is by far the most important determinant of intracavernosal blood flow (Dean and Lue, 2005). During erection, dilation of the resistance arterial bed of the penis provides flow and pressure to the corpora. This is because relaxation of the trabecular smooth muscle allows expansion of the lacunar spaces and trapping of blood by compression of the draining venules (Anderson and Wagner, 1995; Lue and Dahiya, 1997; Udelson et al., 2000). When corpus cavernosum smooth muscle is fully relaxed, the intracavernosal pressure (ICP) is dependent on the cavernosal arterial pressure (Anderson and Wagner, 1995).

Smooth muscle cells are the cellular targets for direct androgenic effects in penile erectile tissues (Mirone et al., 2009). The tone of the trabecular smooth muscle however, is dependent upon a number of other factors including the level of neurotransmitters, hormones, endothelium-derived

factors, adequate expression of receptors, integrity of transduction mechanisms, calcium homeostasis, interaction of contractile proteins and intimate intracellular communication between smooth muscle cells through gap junctions. Alterations in any of these factors may therefore have an implication in the penile smooth muscle function.

The molecular relationship between androgens and penile smooth muscles remains unexplored, but androgens have been shown to induce stem cells to differentiate into muscle lineage instead (Bhasin et al., 2003; Singh et al., 2003). Concordant with this, low androgen levels are associated with impaired myogenic differentiation of mesenchymal progenitor cells, as well as apoptosis of smooth-muscle cells (Liu et al., 2007). This is further supported by the fact that inhibition of 5 α -reductase activity causes stromal remodeling and smooth muscle dedifferentiation (Corradi et al., 2004).

1.2.7 Penile Connective Tissue Morphology

The main connective tissue sheath of the penis, termed the tunica albuginea, encases each of the erectile masses. It is composed of elastic and collagen fibers, and is relatively non-distensible. It is largely responsible for the passive mechanical properties of cavernosal tissue. The tunica of the corpora cavernosa is a bilayered structure that can be divided into an inner circular layer and an outer longitudinal layer (Hsu et al., 1994a; Brock et al., 1997). The inner layer bundles support and contain the cavernous tissue. Radiating from this layer are intracavernous pillars acting as struts, which augment the intercavernous septum and provide essential support to the erectile tissue (Brock et al., 1997). There are numerous trabeculae which cross the corpora cavernosa in all directions and divide them into a series of cavernous spaces (Mirone et al., 2009).

The outer longitudinal layer of the tunica extends from the glans penis to the proximal crura, where it inserts into the inferior pubic ramus (Brock et al., 1997). It aggregates at the tip of the penis to form the distal ligament of the glans (Hsu et al., 2004). This distal ligament, the so called corpora-glans ligament, is a triangular fibrous tissue band connecting the distal blind ends of the two corpora cavernosa with the glans, and acts as a trunk of the glans penis (Hsu et al., 2004). It replaces the os penis that is present in dogs or rats, also termed the baculum, but retains collagen types I and III as common structural and interlocking components, respectively. Without this strong ligament, the glans would be too weak to bear the buckling pressure generated during coitus (Hsu et al., 2004). The relationship between collagen and elastic fibers with hypogonadism remains relatively undescribed.

1.2.8 Penile Vascular System

The penile vascular system consists of vascular spaces (sinusoids) within the penile erectile tissues. The arterial supply of the penis derives from the internal pudendal arteries, which give rise to the deep arteries and the dorsal arteries of the penis. Deep arteries branch to form nutritive and helicine arteries. Nutritive arteries supply oxygen and nutrients to the trabeculae, and helicine arteries empty directly into the cavernous spaces (erectile tissue). There are arteriovenous shunts between the helicine arteries and the deep dorsal vein (Hsu et al., 2003; Junquera and Carneiro, 2005). The lacunar spaces or vascular sinusoids of the corpora cavernosa drain through subtunical veins beneath the tunica albuginea into the emissary veins by way of the deep dorsal vein of the penis. The venous system generally consists of a single deep dorsal vein accompanied by a pair of dorsal arteries positioned between the tunica albuginea and Buck's fascia (Hsu et al., 2003).

The penile sinusoids are lined by endothelial cells and they make the penis to act as a vascular capacitance organ which accumulates blood under systolic pressure (Saenz de Tejada et al., 1991; Udelson et al., 2000). These endothelial cells are targets for androgen hormone activity (Mirone et al., 2009). At vascular level, androgens promote endothelial cell survival and reduce endothelial expression of pro-inflammatory markers (Foresta et al., 2008). Accordingly, hypogonadism is associated with increased risk for atherosclerotic vascular wall remodeling (Demirbag et al., 2005).

1.3 Study Question

What are the structural alterations that occur in the penile architecture involving penile length, smooth muscle, connective tissue morphology and vascular spaces following induced gonadal hormone deficiency?

1.4 Study Justification

A decline in androgen levels in normal aging, androgen deprivation therapy and disorders that either damage the testes or reduce gonadotropin stimulation are known to cause hypogonadism associated with erectile dysfunction (Eunice et al., 2007; Liu et al., 2009). The mechanisms underlying the association between hypogonadism and erectile dysfunction could be structural since the penile architectural integrity is vital in physiology of tumescence and detumescence. This possible anatomical link is fairly underexplored.

1.5 Significance of the Study

Understanding the normal and abnormal penile morphology would improve the basic scientific knowledge of the composition and organization of the penile structures that are thought to play a key role in the mechanism of erection. Knowledge of the structural changes in the erectile tissue may inform new frontiers of investigation and patient follow-up, in addition to providing anatomical basis of understanding the pathophysiology of erectile dysfunction. Alterations may also be important when analyzing tissues from penile biopsy in various gonadal states, and in tissue engineering for phallic grafting (Schultheiss et al., 2004; Patel and Atala, 2011). Understanding the temporal changes that occur in the penis following androgen deprivation may also help to infer time of insult and predict prognosis. In clinical practice, knowledge of pattern of reversal of these changes during androgen therapy may help in treatment monitoring.

1.6 Hypothesis

There is a general decrease in penile length, smooth muscle density, disintegration of connective tissue fibers and collapse of penile vascular spaces by fibrosis and venous leakage following induced gonadal hormone deficiency.

1.7 Study Objectives

1.7.1 Broad Objective

To describe the architectural changes which occur in the rabbit penis following bilateral orchiectomy.

1.7.2 Specific Objectives

To determine and compare the following between normal and castrated rabbits:

- a) Penile length
- b) Distribution and morphology of the smooth muscles in the penile erectile tissues
- c) Quantity and morphology of penile connective tissue fibers (collagen and elastic fibers)
- d) Distribution of fat cells within the penile tissues
- e) Morphology of the cavernosal and corpus spongiosum vascular spaces

CHAPTER 2: MATERIALS AND METHODS

2.1 Study Design and Experimental animals

This was a quasi-experimental study where gonadal hypogonadism was induced by bilateral orchiectomy. Fifteen adult male rabbits obtained from one rabbit farm were used for the study. Nine of these were castrated (intervention group) and six were not (non-intervention group). Five rabbits (3 from intervention and 2 from the non-intervention groups) were perfused three weekly from the time of castration (Table 2).

Table 2: Timing of perfusion after castration (intervention)

GROUP	TIME IN WEEKS				TOTAL
	0 (START)	3	6	9	
INTERVENTION	-	3	3	3	9
NON-INTERVENTION	-	2	2	2	6
TOTAL	-	5	5	5	15

2.1.1 Care of the animals

All the animals were bought from the same farm and the food they were given was the same. After being bought from a rabbit farm where the animals were being fed on vegetables, the animals were kept in separate cages where their regular diet was continued.

2.1.2 Induction of hypogonadism

Hypogonadism was induced by surgical castration under local anesthesia at the beginning of the study in the intervention group using the prescrotal approach. This approach was used because it provides a better anatomical closure of the inguinal canals compared to other techniques of rabbit orchietomy such as traditional scrotal and abdominal approaches (Capello, 2005).

With the animal lying in the dorsal recumbence position under physical restraint, perineal region was identified. The skin was then cleaned with iodine solution then 2 ml of 1% Lignocaine was injected at the prescrotal area and around both scrotal sacs to induce local anesthesia. Due to the position of the rabbit penis, manipulation of the delicate skin of the hemiscrotal sacs, including shaving and scrubbing, is completely avoided in the prescrotal approach. A 2 cm incision was made on the midline just cranial to the base of the hemiscrotal sacs. Blunt dissection of the subcutaneous tissue and inguinal fascia revealed the vaginal processes just before entering the abdomen through the inguinal canal. These were bluntly dissected from the surrounding soft tissues and isolated, and Vicryl 2.0 suture passed around it and tied loosely to act as a stay suture. It was then incised to access the testicle and spermatic cord, which were gently grasped and exteriorized through the incision. The ligament between the hemiscrotal sac and the tail of the epididymis was gently dissected, and the spermatic cord was clamped, ligated and removed en bloc with the testes and epididymis. The preplaced stay suture was then tied to close the vaginal process. The procedure was repeated on the contralateral vaginal process, and skin incision closed using Vicryl 2.0 stitch. The wound was covered by an Elastoplast which all the animals removed at varied times, from immediately to within 24 hours. The animals were allowed a period of three

weeks, six weeks and nine weeks for possible structural alterations to establish due to the induced hypogonadal state.

2.1.3 Perfusion and fixation

At the end of each defined period, three castrated rabbits and two non-castrated rabbits were anaesthetized with inhaled chloroform. In the supine position, the thoracic region was identified and the costal cartilages cut bilaterally to remove the sternum. The pericardium was identified and split to expose the heart. The apex of the left ventricle was identified and punctured with a needle fitted with normal saline drip under gravity. The right atrium was punctured to create an outflow point. The animal died during this process, evidenced by the loss of corneal reflex and apex beat. Once the blood had been replaced by normal saline, 10% formal saline was infused through the same route. Penile tissues were harvested and cut into smaller pieces before being immersed in 10% formal saline in specimen bottles.

2.2. Tissue Processing for Light Microscopy

The tissues were fixed for a period of at least 24 hours in 10% formal saline. They were then dehydrated in ascending grades of ethyl alcohol, starting with 70% alcohol to absolute alcohol. Toluene was used as clearing agent. Embedding was done in fresh molten wax and mounted on wooden blocks. A sledge microtome (Leica® Model SM2400, Germany) was used to produce 7 micron thick sections. These were then floated in a warm water bath then fixed on glass slides and allowed to air dry for at least 12 hours. Dewaxing was then done followed by rehydration to different levels depending on the stain.

2.3 Determination of specific penile parameters

The penile length, distribution and morphology of smooth muscles, adipocytes, collagen fibers and elastic fibers and morphology of penile vascular spaces were determined as follows:

2.3.1 Measurements of penile length

The penile lengths were measured using a digital Vernier caliper (accuracy 0.5mm). This was from the palpable lower border of the pubic symphysis (pubopenile junction) to the tip of the glans penis (Wessells et al., 1996; Dillon et al., 2008). These measurements were taken for each animal at the beginning, and every three weeks until the specific rabbit was perfused. The three weekly measurements were taken just before the perfusion. The values were recorded for each rabbit to study the trend. The mean lengths of each rabbit category was determined and compared to the mean lengths of other rabbit categories.

2.3.2 Determination of the distribution and morphology of penile smooth muscles

After rehydration of the tissue sections in descending grades of alcohol concentration, the tissue sections were stained with Mason's trichrome to illustrate smooth muscles. These were reddish. Their location within the penile trabeculae were noted and the density determined by stereological technique. All values were recorded and compared with sections from other rabbit groups.

2.3.3 Determination of collagen fiber distribution and morphology

Collagen fibers were demonstrated in tissues stained with Mason's trichrome where they stained green. The purpose of the Masson's trichrome stain is primarily to demonstrate collagen and muscle in tissues. The protocol involves the use of three stains namely: (i) iron hematoxylin which

stains the nuclei of cells; (ii) light green which stains collagenous green; and (iii) Ponceau-Acid Fuchsin with Phosphotungstic Acid as a mordant stains for the muscles red. The orientation, organization and continuity of collagen bundles were noted. Their quantity was determined by the stereological techniques.

2.3.4 Determination of the distribution of elastic fibers

After rehydration of the tissue sections up to 95% alcohol, the tissue sections were stained with Weigert's resorcin-fuchsin stain to demonstrate elastic fibers. The method is based on the affinity towards elastic fibers displayed by resorcin-fuchsin, a precipitate resulting from a reaction between resorcin and basic fuchsin and ferric chloride. Their location within the penile tissues were noted and the density determined by stereological technique described. These were compared with sections from different rabbit groups.

2.3.5 Determination of fat cell distribution

Fat cells were demonstrated in the tissue section after staining with Mason's trichrome. They appeared as clusters of non-staining cells within the penile tissues. They were differentiated from the penile vascular spaces by the presence of their nuclei. Their presence, location and quantity were determined.

2.3.6 Determination of penile vascular spaces

The vascular spaces were identified in stained sections as empty spaces within the penile trabecular, surrounded by the trabeculae. Their sizes were determined by stereological techniques already described, recorded and compared with other rabbit groups.

2.4 Stereology

Stereological techniques were used in morphometric analysis of functional components –vascular volume in corpus cavernosum and corpus spongiosum, smooth muscle density and connective tissue fiber components. Smooth muscle and connective tissue density was done using the Cavalieri principle of point counting (Mandarim-de-Lacerda, 2003) [Figure 1] and data expressed as volume densities (%). From each rabbit penis, ten different sections were selected from ten fragments. Then, five random fields of corpus cavernosum were evaluated from each section. There were, therefore, fifty (50) test areas from each penis. All images were photographed with a digital camera directly coupled to a microscope, at X400 of magnification, and projected to a monitor screen.

Following technique described by Gundersen et al., (1988) and Bancroft and Cook (1994) and also used by Costa et al (2009; 2010), the selected histological areas were analyzed using a superimposed 42-point grid on the digital images on the monitor screen. Such a grid system produces lines and point probes regularly arranged, which are used to facilitate estimation of the specific tissue densities (Mandarim-de-Lacerda, 2003). The volumetric densities (V_v) of the histological structures were evaluated while unaware of the source of the tissue samples.

From stereological principles in isotropic tissue, the distribution area of a specific structure, as determined on a two-dimensional section of a structure, is proportional to the volume distribution of the structure (Pinheiro et al., 2000; Mandarim-de-Lacerda, 2003). The volume density of the histological components was calculated by the formula $V_v = P_p/P_t$, where V_v is the volume density, p is the tissue component under consideration (smooth muscle, collagen or elastic fibers), P_p is the number of test points associated with p , and P_t is the total number of points of the test

system. The ratios of smooth muscle and connective tissue components were estimated from the densities of each component. Thickness of smooth muscle cells and connective tissue fibers was done by point sampled intercepts (Figure 2).

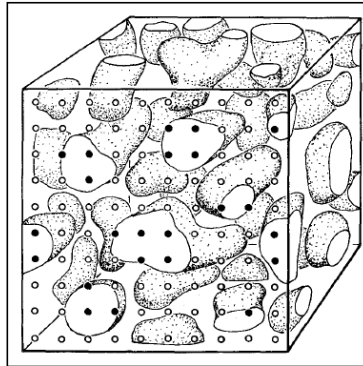


Figure 1: Cavalieri Principle of point counting.

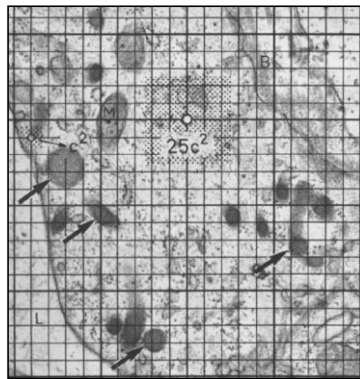


Figure 2: Point sampled intercepts method of determining thickness

2.5 Data Analysis

The data was coded and analyzed by computer software, Statistical Package of Social Sciences (SPSS) version 17.0. The Student's t test was used for mean comparisons of the penile lengths and the volumetric densities of each of the elements of the erectile tissues (smooth muscles, collagen and elastic fibers and vascular spaces). A p-value of less than 0.05 was considered statistically significant.

CHAPTER 3: RESULTS

A number of changes were observed in the castrated rabbits. These were noted to correlate with the duration of exposure to hypogonadism. These changes included reduction in the non-erect penile length, trabecular smooth muscle cells and elastic fibers. There was erectile tissue fibrosis with increased collagen: smooth muscles ratios. Disruption of the lamella arrangement of collagen fibers of the tunica albuginea and disintegration of the inter-cavernosal septum were also observed. There was cavernosal artery fibrosis, leakage of red blood cells due to reduced endothelial integrity, and general narrowing or collapse of subtunical vessels in the castrated group. Progressive fat cell accumulation in the subtunical and intratunical zones was reported.

The normal penile sections consisted of two corpora cavernosa dorsally and one corpus spongiosum ventrally. These were covered by a thick connective tissue capsule, the tunica albuginea (Figure 3A-B). The corpora cavernosa were separated by the intercavernosal septum, which is an extension of the tunica albuginea. The tunica albuginea surrounding the corpora cavernosa was thicker compared to that of the corpus spongiosum, and both were well defined and separated from the erectile tissues (Figure 3B). The cavernosal tunica albuginea consisted of two layers: an outer longitudinal layer of thick collagenous fibers and an inner circular layer of undulating collagen fiber bundles, arranged in concentric lamellae (Figure 3C-D). Each bundle was composed of densely packed collagen fibers running parallel to each other.

The penile erectile tissues showed a honeycomb appearance on cross-sections. Each vascular space was surrounded by a trabecular network consisting of smooth muscles, collagen and elastic fibers (Figure 3E-H). There were thick bundles of smooth muscle cells (Figure 3G) and elastic fibers (Figure 3H) in the trabeculae.

Figure 3A-H: Normal Penile Structure

Figure 3A: Cross sectional structure of the penile corpora cavernosa of a normogonadic rabbit. Note the tunica albuginea (TA) forming a thick capsule around the two corpora cavernosa (CC). The two corpora are separated by the intercavernosal septum (ICS). Stain = Masson's Trichrome; Magnification = X40.

Figure 3B: Structure of the penile corpus spongiosum of a normogonadic rabbit. The corpus spongiosum (CS) is surrounded by the tunica albuginea (TA1). The penile urethra (U) traverses the corpus spongiosum. The tunica albuginea (TA2) covering the corpus cavernosum (CC) was noted to be thicker compared to the one covering the corpus spongiosum (TA1). Both were well defined and separated from the erectile tissues. Stain = Masson's Trichrome; Magnification = X40.

Figure 3C: Structure of the cavernosal tunica albuginea of a normogonadic rabbit penis. Note the inner circular layer of collagen fibers (IL) arranged in concentric lamellae and an outer longitudinal layer (OL). CC = Corpus cavernosum; Stain = Masson's Trichrome; Magnification = X100.

Figure 3D: Structure of the inner circular layer of the cavernosal tunica albuginea of a normogonadic rabbit. Note the wavy/undulating and continuous arrangement of the fibers. Stain = Masson's Trichrome; Magnification = X400.

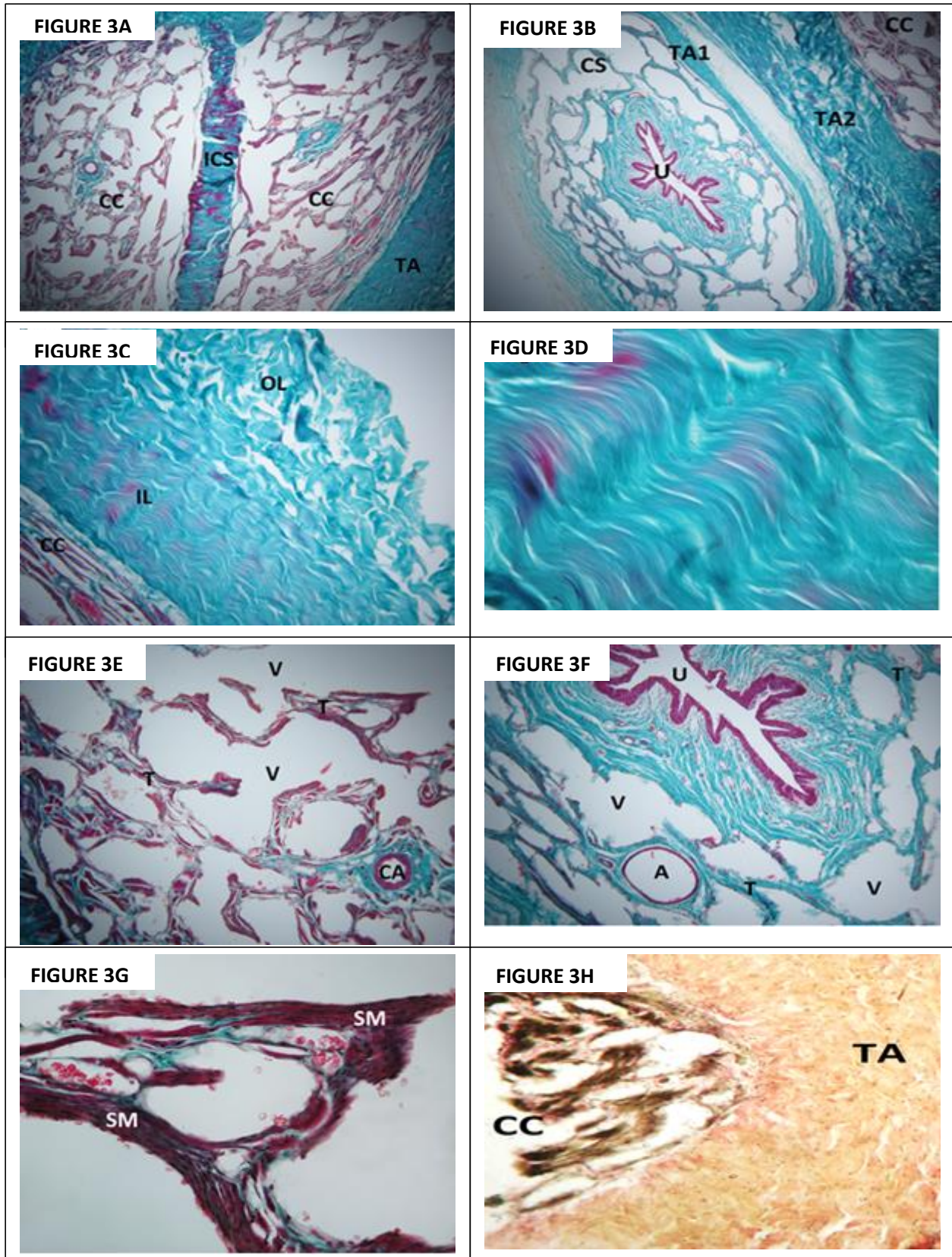
Figure 3E: Structure of the penile corpus cavernosum of a normogonadic rabbit. Note the honeycomb appearance on cross-section, formed by the trabecular network (T) surrounding large vascular spaces (V). Note also components of the trabecular network namely the smooth muscles (stained red) and collagen fibers (stained green). CA = Cavernosal artery; Stain = Masson's Trichrome; Magnification = X100.

Figure 3F: Structure of the penile corpus spongiosum of a normogonadic rabbit. Note the trabecular network (T) surrounding large vascular spaces (V). A = Artery; U = Penile Urethra; Stain = Masson's Trichrome; Magnification = X100.

Figure 3G: Structure of the trabecular system of the penile corpus cavernosum of a normogonadic rabbit. Note the thick bundles of smooth muscle (SM) and the thin collagenous strands between the smooth muscles. Stain = Masson's Trichrome; Magnification = X400.

Figure 3H: Structure of the penile corpus cavernosum of a normogonadic rabbit. Note the numerous elastic fiber (stained black) bundles within the corpus (CC). Stain = Weigert's; Magnification = X100.

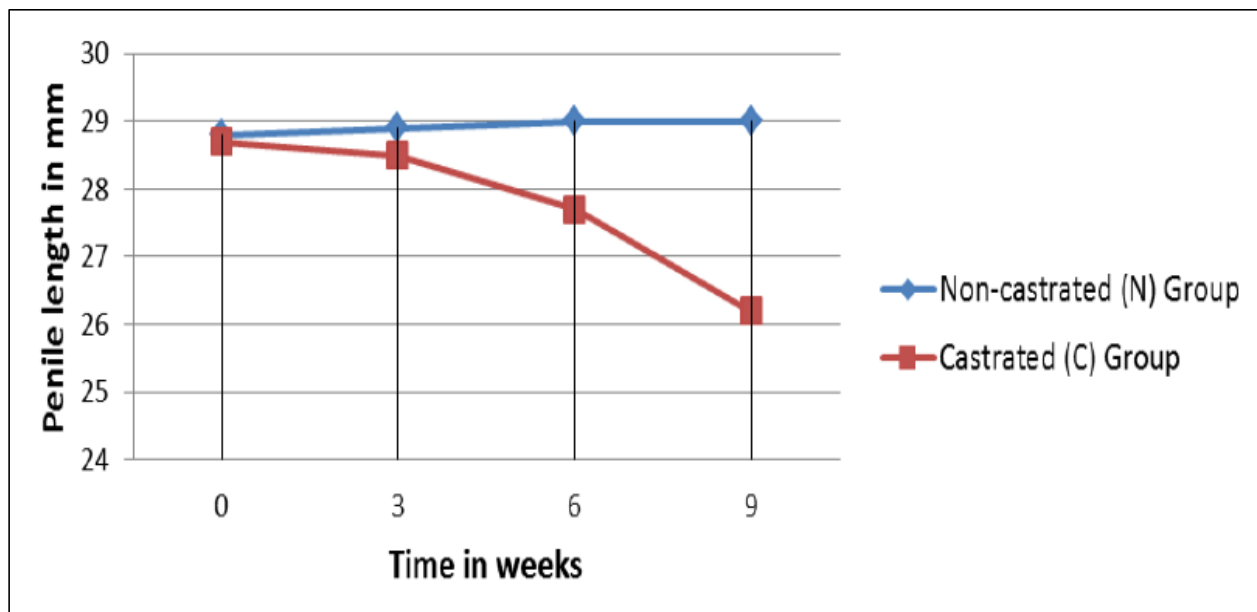
Figure 3A-H: Normal Penile Structure



3.1 Penile Length

The mean length of the non-erect penis at the beginning of the study was 28.7 mm and 28.8 mm for castrated and non-castrated groups respectively. These ranged between 27mm and 30mm. There was an average reduction in the non-erect penile length by 0.7%, 3.4% and 8.7% in the castrated group at the end of the third, sixth and ninth week respectively (Figure 4). The reduction in penile length was statistically significant (p -value < 0.05), and was more marked in rabbits that were exposed to hypogonadism over a longer period of time. The penile length in the non-castrated group remained fairly constant during the study period, demonstrating percentage increase in length by 0.4%, 0.7% and 0.7% in the third, sixth and ninth week respectively.

Figure 4: Comparisons of the penile length in Castrated and Non-castrated Rabbits over time



3.2 Penile Smooth Muscle Cells

Tissue sections from control animals typically exhibited abundant areas of dense trabecular smooth muscle (Figure 3A). These cells had discrete localization in the trabeculae, forming a sub-endothelial layer that surrounds the vascular spaces within the erectile tissues. Both longitudinally and transversely oriented bundles of cells were noted, but the latter were more common (Figure 3G). Androgen ablation resulted in reduced trabecular smooth muscle content and increased connective tissue, as determined by Masson trichrome staining and stereology (Figures 5A-D). Maximal reduction in smooth muscle components was seen in the castrated rabbits that were perfused after nine weeks (Figures 5E and 5F). Notably, the proportion of collagenous fibers to smooth muscles in the trabecular increased significantly, maximal at nine weeks (Figure 5E). The thickness of smooth muscle bundles in the penile trabeculae also decreased with a longer exposure to hypogonadal state, being most marked at nine weeks after castration.

Figure 5A-F: Smooth Muscle Morphology in Castrated rabbit

Figure 5A: Structure of the penile corpus cavernosum of a rabbit three weeks after castration. Notice the increased quantity of collagenous fibers intermingled with smooth muscle cells (arrows) within the trabeculae compared to the normogonadic rabbits. TA = Tunica albuginea; Stain = Masson's Trichrome; Magnification = X100.

Figure 5B: Structure of the penile cavernosal trabecular of a rabbit three weeks after castration. There was still a significant amount of smooth muscles (stars) in the penile trabecular. Of note also is the increase in the collagenous fibers intermingled with smooth muscle cells within the trabeculae compared to the normogonadic rabbit penis. Stain = Masson's Trichrome; Magnification = X400

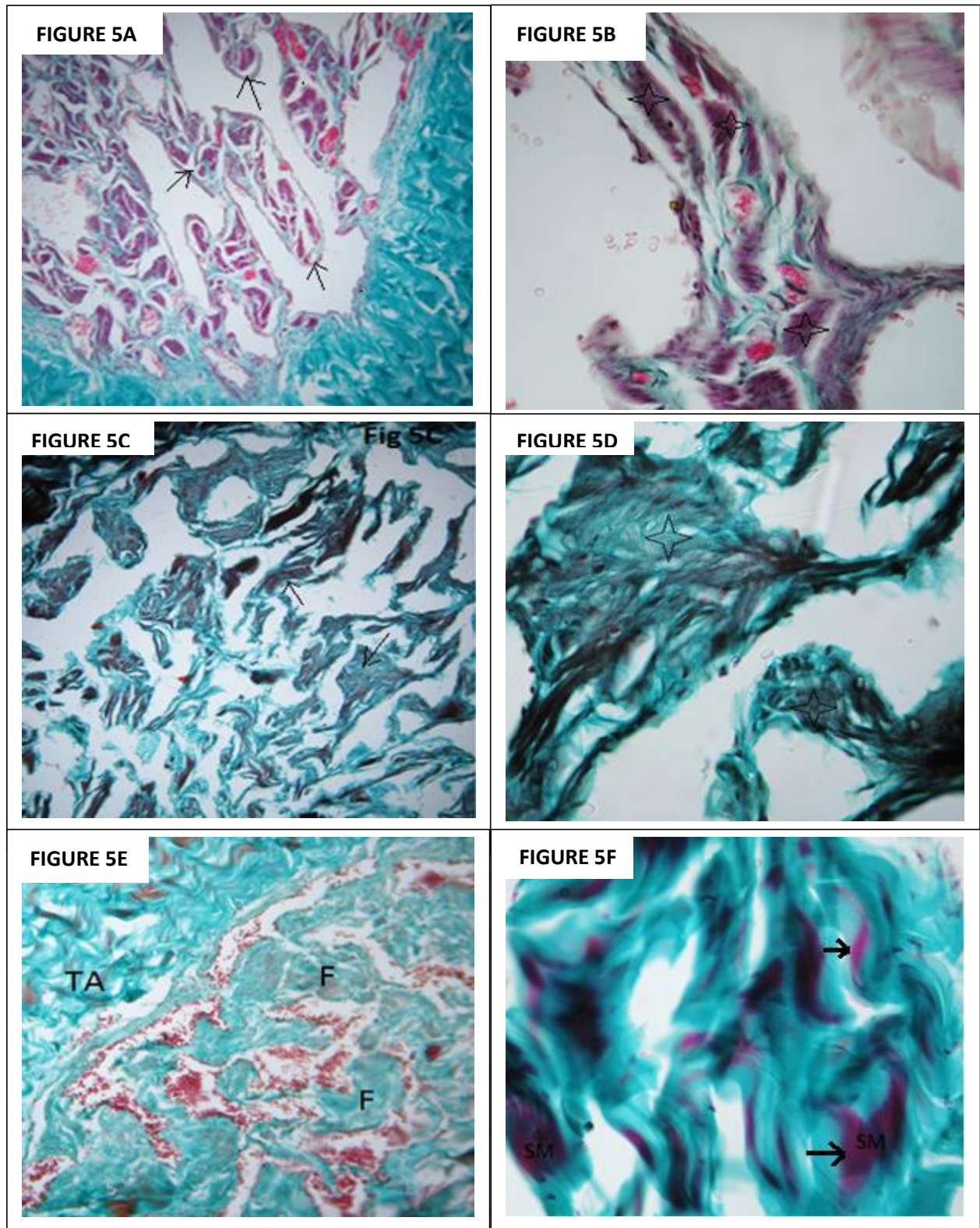
Figure 5C: Structure of the penile corpus cavernosum of a rabbit six weeks after castration. There is less smooth muscle cells (arrowed) in the penile trabeculae, and the collagen: smooth muscle ratio is higher compared to normogonadic rabbits. Stain = Masson's Trichrome; Magnification = X100.

Figure 5D: Structure of the penile cavernosal trabecular of a rabbit six weeks after castration. There were thin smooth muscle bundles within the penile trabeculae (starred). Notably, the collagen: smooth muscle ratio was higher compared to normogonadic rabbits. Stain = Masson's Trichrome; Magnification = X400.

Figure 5E: Structure of the penile corpus cavernosum of a rabbit nine weeks after castration. There was hardly any smooth muscle cells in the penile erectile tissues. Instead, there was marked cavernosal fibrosis (F). TA = Tunica albuginea; Stain = Masson's Trichrome; Magnification = X100.

Figure 5F: Structure of the penile cavernosal trabecular of a rabbit nine weeks after castration. There was scattered smooth muscle cells (arrows) within the trabeculae. Of note also is the marked cavernosal fibrosis. Stain = Masson's Trichrome; Magnification = X400.

Figure 5A-F: Smooth Muscle Morphology in Castrated rabbits



3.3 Penile Connective Tissue fiber Morphology

3.3.1 Morphology of the collagenous fibers of the tunica albuginea

The tunica albuginea surrounding the corpus cavernosum was made up almost entirely of thick collagen fibers (Figure 3C). In normogonadic rabbit the tunica albuginea was made up of undulating bundles of collagenous fibers which were arranged in outer longitudinal and inner circular layers (Figures 3C and 3D). In both layers, several bundles were successively stacked to show a conspicuous lamellar arrangement, which was more definite in the inner circular layer (Figure 3D). From the inner circular layer of the tunica albuginea finger-like trabeculae penetrated inward. They appeared as thin and thick irregular strands around the cavernous spaces. These trabeculae were predominantly composed of smooth muscle fibers which were partly separated from the cavernous spaces by collagen fibers in the normogonadic rabbits.

In the hypogonadic rabbits, notable progressive changes in the connective tissue profile of the tunica albuginea included the disruption of the lamella arrangement of collagen fibers (Figures 6A-F). The disruption was less severe in rabbits exposed to hypogonadism for three weeks (Figure 6A and 6B) but was more prominent in rabbits exposed to hypogonadism over six (figures 6C and 6D) and nine weeks (Figures 6E and 6F). The inner circular layer of collagen fibers were observed to be arranged in lamellae formed by continuous bundles of thick collagenous fibers (Figure 3D). In the hypogonadic rabbits however, the changes ranged from simple separation of the collagenous layers, evidenced by wider spaces between each lamella, to actual discontinuity within each lamellar system (Figures 6D and 6E).

Figure 6A-F: Structural changes in the collagen fiber arrangement of the tunica albuginea

Figure 6A: Structure of the penile cavernosal tunica albuginea of a hypogonadic rabbit seen after 3 weeks of castration, also showing an inner circular layer of collagen fibers (TA-1) arranged in concentric lamellae and an outer longitudinal layer (TA-2). This structure is almost similar to the one in normogonadic rabbits, but there are wider spaces between the collagenous bundles, an earlier evidence of fiber disruption. CC = Corpus cavernosum; Stain = Masson's Trichrome; Magnification = X100.

Figure 6B: Structure of the penile cavernosal tunica albuginea of a hypogonadic rabbit seen after 3 weeks of castration, showing some separation of the inner circular layer of collagen fibers. The collagen bundles are however continuous as in the normogonadic rabbits; Stain = Masson's Trichrome; Magnification = X400.

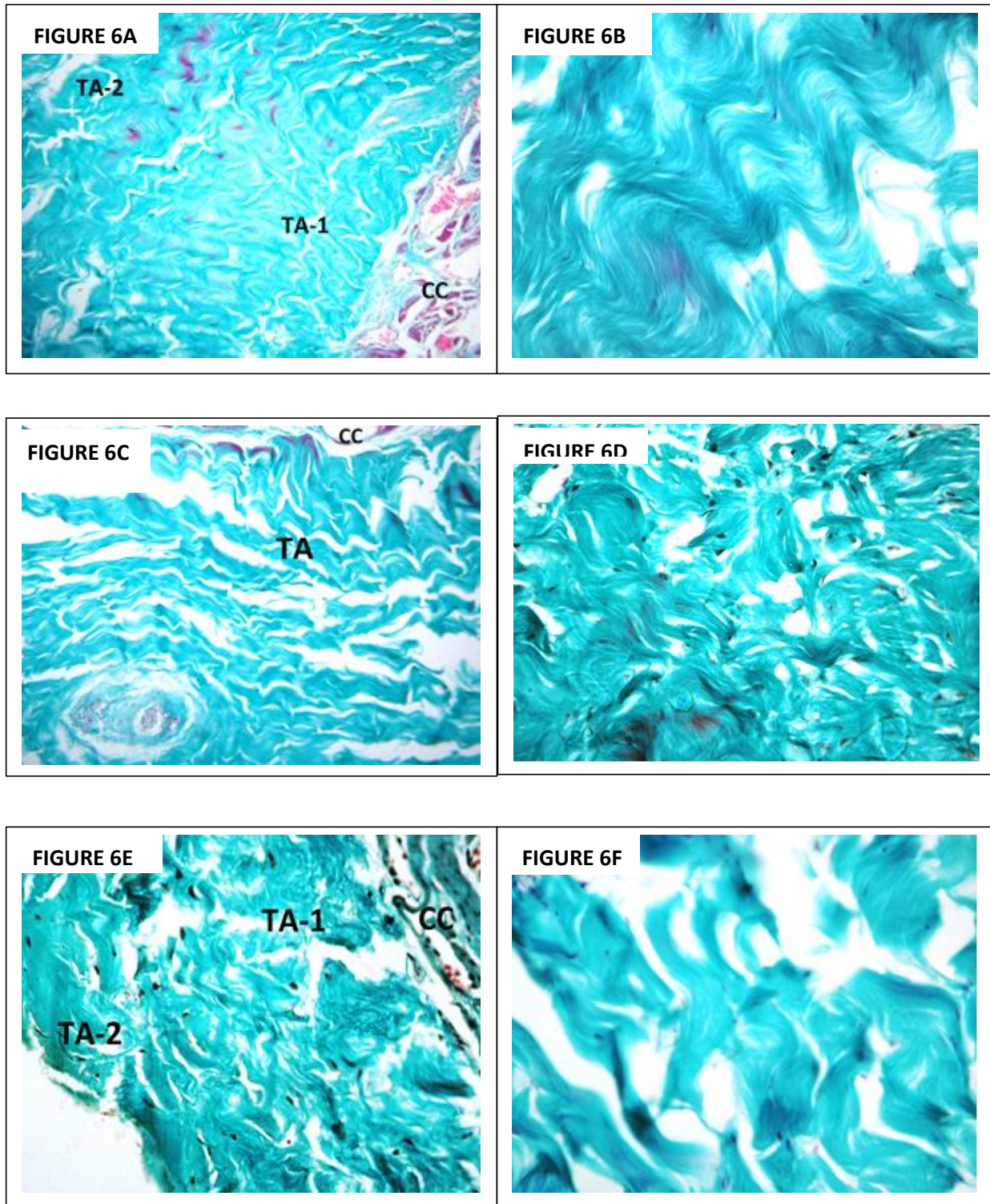
Figure 6C: Structure of the penile cavernosal tunica albuginea of a hypogonadic rabbit seen after 6 weeks of castration. There is notable disintegration in the inner circular layer of collagen fibers (TA) with wider spaces between the collagenous bundles. CC = Corpus cavernosum; Stain = Masson's Trichrome; Magnification = X100.

Figure 6D: Structure of the penile cavernosal tunica albuginea of a hypogonadic rabbit seen after 6 weeks of castration, showing moderate disintegration in the inner circular layer of collagen fibers with wider spaces between the collagenous bundles.; Stain = Masson's Trichrome; Magnification = X400.

Figure 6E: Structure of the penile cavernosal tunica albuginea of a hypogonadic rabbit seen after 9 weeks of castration. There is definite disintegration of collagenous fibers in both the inner circular layer (TA-1) and the outer longitudinal layer (TA-2). Also noted was the discontinuity of the collagenous bundles. CC = Corpus cavernosum; Stain = Masson's Trichrome; Magnification = X100.

Figure 6F: Structure of the penile cavernosal tunica albuginea of a hypogonadic rabbit seen after 9 weeks of castration, showing definite disintegration of collagenous fibers. Note the discontinuous morphology of the collagenous bundles; Stain = Masson's Trichrome; Magnification = X400.

Figure 6A-F: Structural changes in the collagen fiber arrangement of the tunica albuginea



3.3.2 Morphology of the intercavernosal septum

The intercavernosal septa, predominantly made of collagen fibers, was also disintegrated in the intervention group but preserved in the non-intervention group. The septum was seen to be continuous in the normogonadic rabbits (Figure 7A), but this continuity was disturbed proportional to the period of hypogonadism (Figure 7B-D). After nine weeks of castration, there was actual degeneration of the septum (Figure 7D).

Figure 7A-D: Structural Changes in the intercavernosal septum

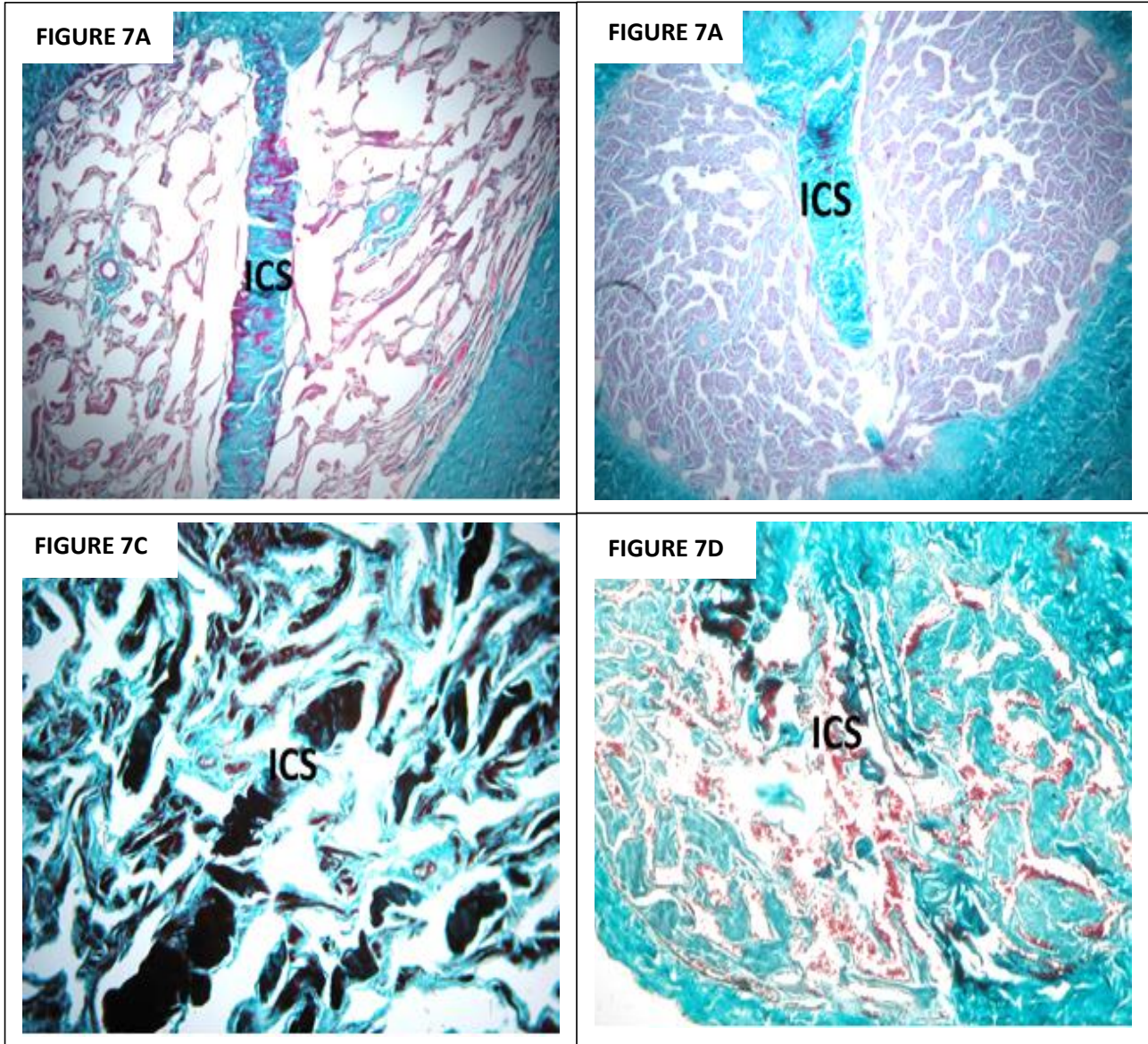
Figure 7A: Structure of penile corpora cavernosa of a normogonadic rabbit showing the intercavernosal septum. In this group the intercavernosal septum (ICS) is intact and continuous; Stain = Masson's Trichrome; Magnification = X40.

Figure 7B: Structure of penile corpora cavernosa of a hypogonadic rabbit seen three weeks after castration to show the intercavernosal septum (ICS). The intercavernosal septum was relatively intact and continuous; Stain = Masson's Trichrome; Magnification = X40.

Figure 7C: Structure of penile corpora cavernosa of a hypogonadic rabbit seen six weeks after castration to show the intercavernosal septum (ICS). There was moderate disintegration of the septum (ICS); Stain = Masson's Trichrome; Magnification = X40.

Figure 7D: Structure of penile corpora cavernosa of a hypogonadic rabbit seen nine weeks after castration to show the intercavernosal septum (ICS). There was significant degeneration of the intercavernosal septum (ICS); Stain = Masson's Trichrome; Magnification = X40.

Figure 7A-D: Structural Changes in the intercavernosal septum



3.3.3 Morphology of the trabecular systems of the erectile tissues

Penile trabecular contained collagenous fibers occurring in thin bundles which extended from the inner circular layer of the tunica and showed a wavy arrangement in the normogonadic rabbits (Figures 3A and 3D).

Orchiectomy resulted in reduced trabecular smooth muscle and increased collagen fiber content in both corpora cavernosa and corpus spongiosum (Figures 5). This was more evident in the hypogonadic rabbits after six and nine weeks (Figure 8A and 8B respectively), characterized by marked fibrosis, reduced smooth muscle density and collapse of vascular spaces.

The corpus spongiosum also displayed progressive fibrosis of the erectile tissue with increasing duration of hypogonadism (Figures 9A-D). Furthermore, spongiosal tunica albuginea, initially well delineated from the enclosed erectile tissue, was progressively fused with the general trabecular fibrosis (Figure 9C-D). Erectile tissue fibrosis was observed more in the corpus spongiosum than corpus cavernosum.

Figure 8A-B: Cavernosal trabecular morphology

Figure 8A: Structure of the penile cavernosal trabecular in a hypogonadic rabbit six weeks after castration. Observe the relatively high quantity of collagenous fibers (F) within the cavernosal trabecular compared to smooth muscles (arrows). ICS = Intercavernosal septum; Stain = Masson's Trichrome; Magnification = X100.

Figure 8B: Structure of the penile cavernosal trabecular in a hypogonadic rabbit nine weeks after castration. The most notable thing here is the generalized cavernosal fibrosis (F), and vascular leakage evidenced by numerous red blood cells (arrows) within the trabeculae. TA = Tunica albuginea; Stain = Masson's Trichrome; Magnification = X100.

Figure 8A-B: Cavernosal trabecular morphology

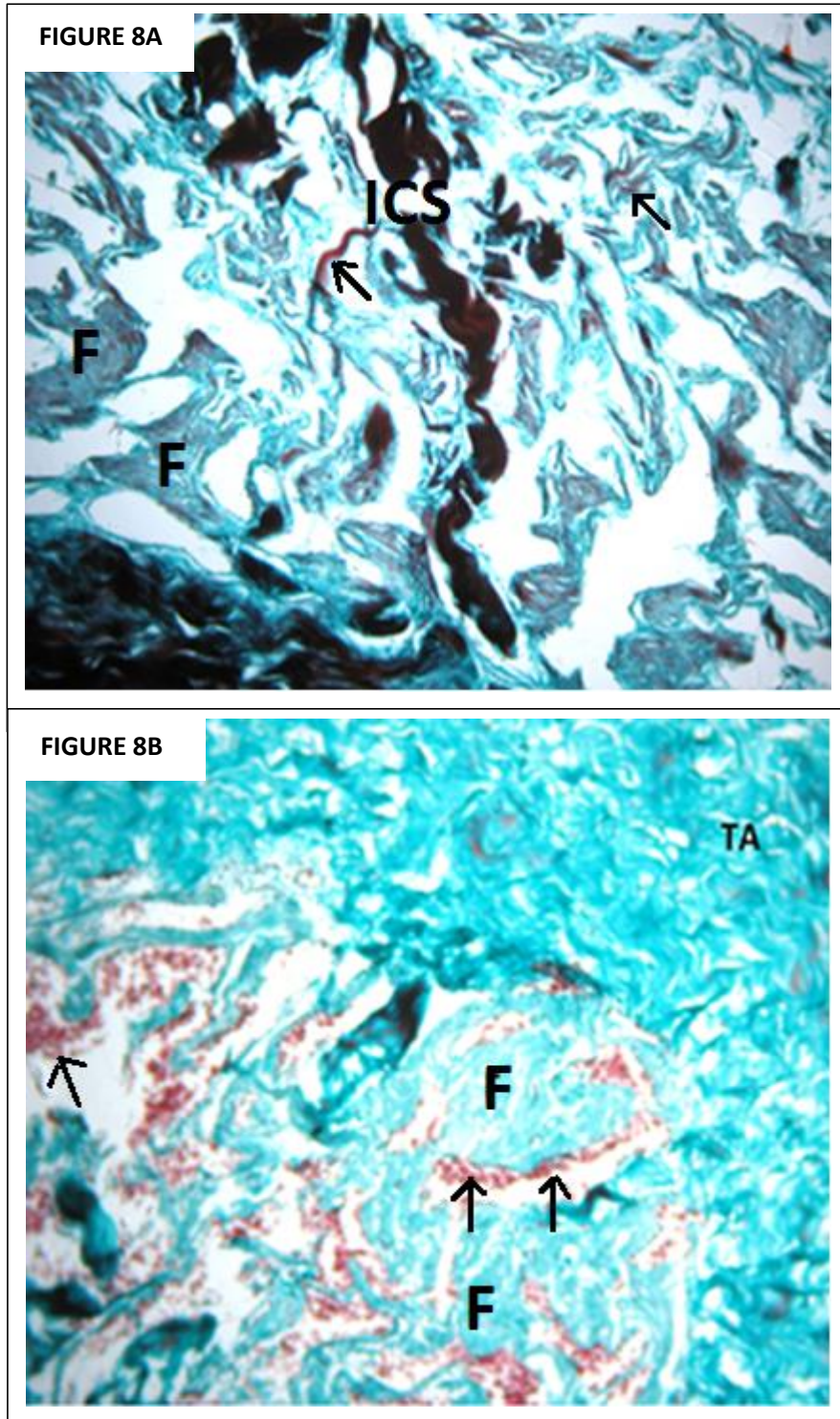


Figure 9A-D: Spongiosal Trabecular Morphology

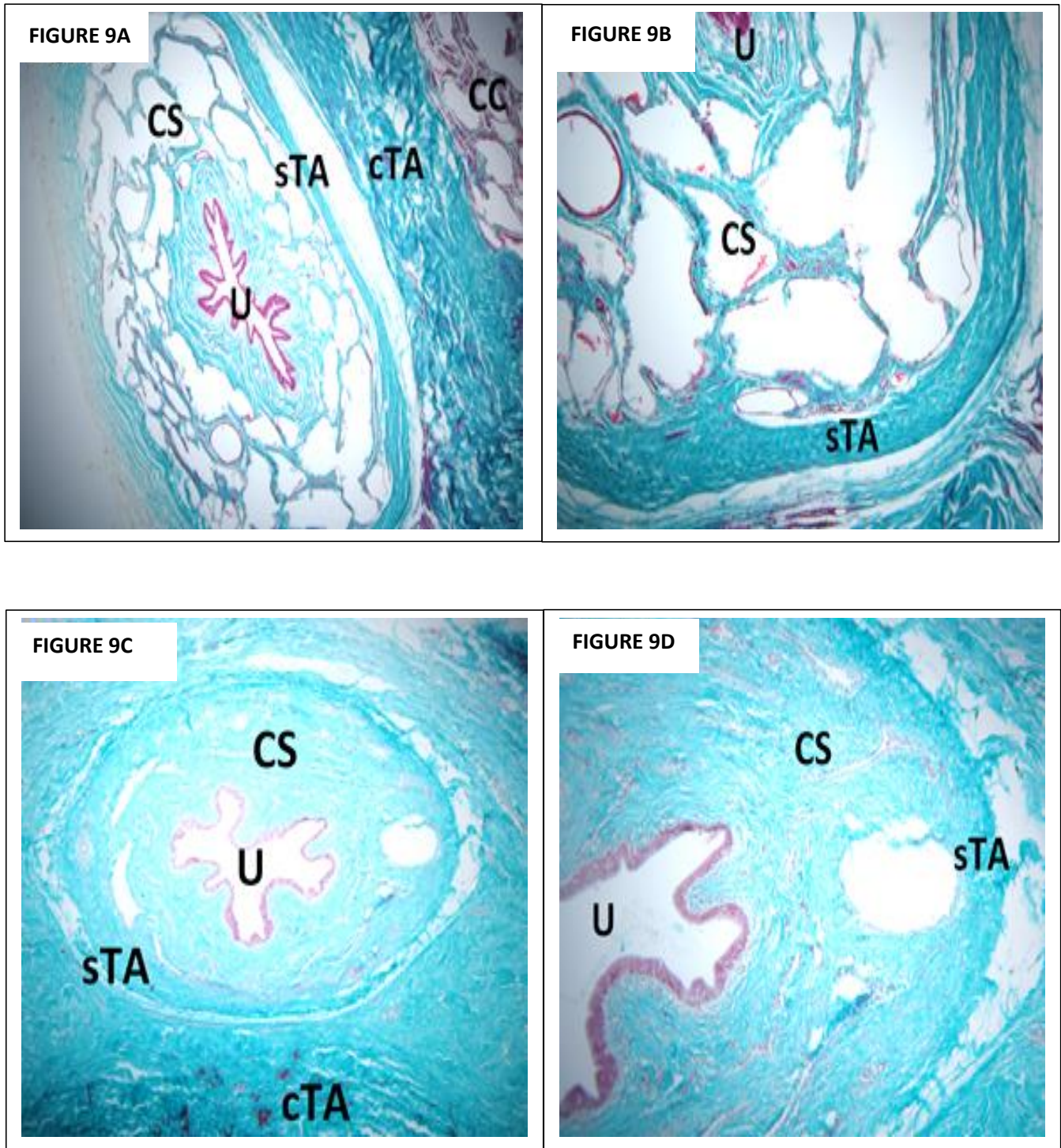
Figure 9A: Structure of the penile corpus spongiosum in a normogonadic rabbit. The corpus spongiosum (CS) morphology shows large vascular spaces with high quantity of smooth muscles and less collagenous fibers within the trabecular. U = Penile Urethra; CC = Corpus cavernosum; sTA = Spongiosal tunica albuginea; cTA = Cavernal tunica albuginea. Stain = Masson's Trichrome; Magnification = X40.

Figure 9B: Structure of the penile corpus spongiosum in a normogonadic rabbit. The corpus spongiosum (CS) morphology shows large vascular spaces with high quantity of smooth muscles and less collagenous fibers within the trabecular. Also note a well demarcated spongiosal tunica albuginea (sTA). U = Penile Urethra; Stain = Masson's Trichrome; Magnification = X100.

Figure 9C: Structure of the penile corpus spongiosum in a hypogonadic rabbit seen nine weeks after castration. The corpus spongiosum (CS) morphology shows marked fibrosis with diminished vascular spaces and smooth muscle density. U = Penile Urethra; sTA = Spongiosal tunica albuginea; cTA = Cavernal tunica albuginea; Stain = Masson's Trichrome; Magnification = X40.

Figure 9D: Structure of the penile corpus spongiosum in a hypogonadic rabbit seen nine weeks after castration. The corpus spongiosum (CS) morphology showing marked fibrosis with diminished vascular spaces and smooth muscle density. Note fusion of the spongiosal tunica albuginea (sTA) with the general spongiosal fibrosis. U = Penile Urethra; Stain = Masson's Trichrome; Magnification = X100.

Figure 9A-D: Spongiosal Trabecular Morphology



3.3.4 Elastic Fiber Morphology

Weigert's resorcin-fuchsin staining of sections of the tunica albuginea showed minimal or no elastic fibers of normal and hypogonadic rabbits respectively. In the normogonadic rabbits, elastic fibers were seen to be between the collagenous bundles. Numerous elastic fibers were however observed in the corpus cavernosum, corpus spongiosum and glans penis both as a loose and fine meshwork of branching fibrils and in bundles. These fibers had tortuous profile and mostly surrounded sinusoids. At the periphery of the corpus cavernosum the fibers were parallel to the long axis of the penis, while in deeper regions there was an irregular orientation. In both normogonadic and hypogonadic rabbits, these fibers were seen in less quantity in corpus cavernosum than in the corpus spongiosum, and even more in the glans penis. The amount of these fibers in the cavernosal trabecular system decreased significantly with duration of hypogonadism, being seen most in the normogonadic rabbits and least in at nine weeks after castration.

3.4 Fat cell accumulation

Histologic examination of Masson's trichrome-stained penile tissue sections from intact animals showed normal tissue morphology with a characteristic network of cavernosal spaces (sinusoids) and trabecular smooth muscle bundles surrounded by an intact tunica albuginea (Figure 3). In contrast, penile tissue sections from orchietomized rabbits and examined after a period of six and nine week exhibited clusters of adipocytes in the subtunical region of the corpus cavernosum (Figures 10A, 10B and 10C). The quantity and distribution of these fat containing cells was greater in penile tissue sections after nine weeks of hypogonadism. Furthermore, in one rabbit exposed to hypogonadism for nine weeks, there was fat cell accumulation within the tunica albuginea (Figure 10D). None of these cells was seen in the normogonadic rabbits or in the rabbits exposed to hypogonadism for three weeks.

Figure 10A-D: Fat Cell Accumulation

Figure 10A: Structure of penile corpus cavernosum of a hypogonadic rabbit six weeks after castration showing accumulation of fat cells (arrowed) in the sub-tunical zone. CC = Corpus cavernosum; TA = Tunica albuginea. Stain = Masson's Trichrome; Magnification = X40.

Figure 10B: Structure of penile corpus cavernosum of a hypogonadic rabbit six weeks after castration showing accumulation of fat cells (FC) in the sub-tunical zone. TA = Tunica albuginea. Stain = Masson's Trichrome; Magnification = X100

Figure 10C: Structure of penile corpus cavernosum of a hypogonadic rabbit nine weeks after castration showing marked accumulation of fat cells (FC) in the sub-tunical zone. TA = Tunica albuginea. Stain = Masson's Trichrome; Magnification = X100

Figure 10D: Structure of cavernosal tunica albuginea of hypogonadic rabbit penis nine weeks after castration showing accumulation of fat cells (FC) within the tunica albuginea (TA). Stain = Masson's Trichrome; Magnification = X100

Figure 10A-D: Fat Cell Accumulation

FIGURE 10A

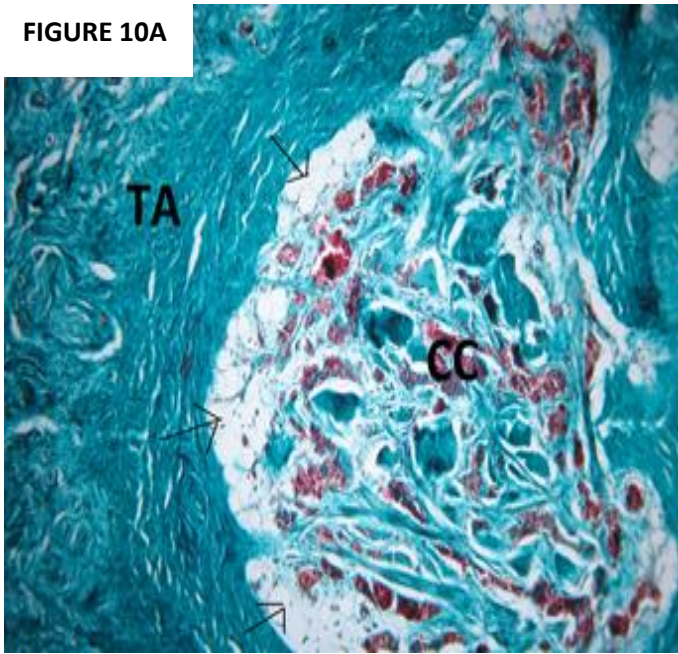


FIGURE 10B

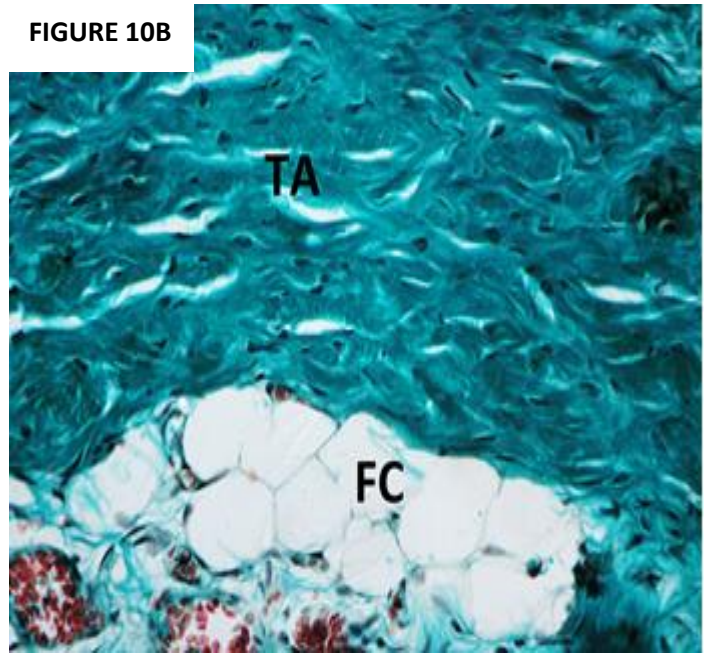


FIGURE 10C

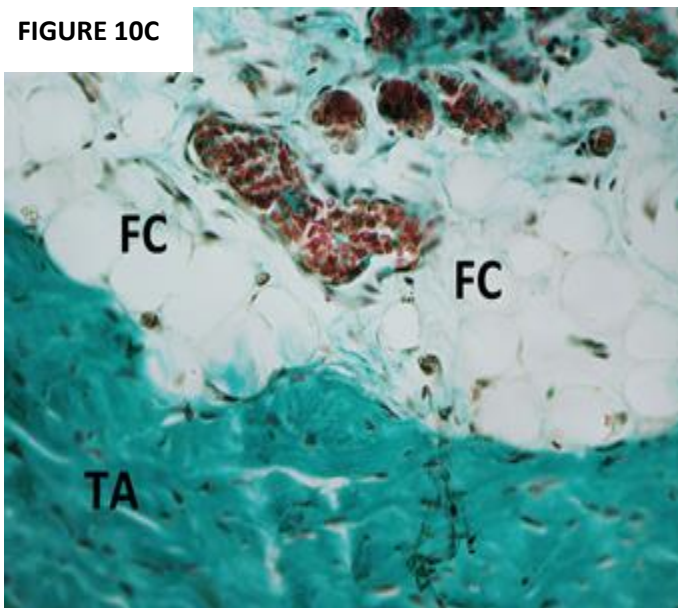
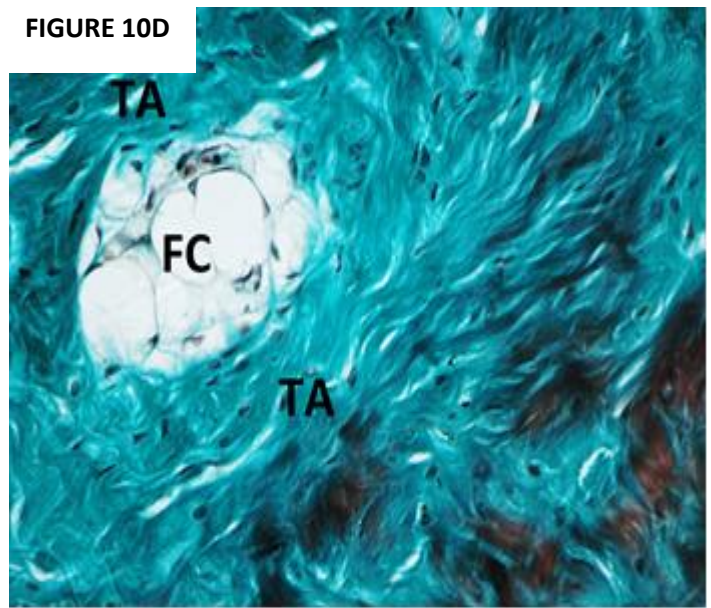


FIGURE 10D



3.5 Penile erectile tissue vascular spaces

The vascular spaces of both corpus cavernosum and corpus spongiosum from castrated rabbit groups were noted to be progressively collapsed (Figure 11) compared to the non-castrated group (Figures 3E and 3F), proportionate to the duration of hypogonadism. There were minimal vascular spaces in the erectile tissue trabeculae, evidenced by direct observation and by stereology. In the rabbits that were exposed to nine weeks of hypogonadism, there was notable red blood cell leakage into the cavernosal trabecular tissues, signifying loss of endothelial integrity (Figure 11E).

The tunica media of the cavernosal artery, being predominantly made of smooth muscle cells, was prominent in the non-castrated rabbit group, but it was almost lacking in the castrated group at the end of nine weeks of hypogonadism (Figures 12A and 12B). Furthermore, there was significant collapse of the sub-tunical vessels in the intervention group, while their integrity was relatively preserved in the non-castrated rabbits (Figure 12C and 12D).

Figure 11A-F: Morphology of cavernosal and Spongiosal vascular spaces in castrated rabbits

Figure 11A: Structure of the penile corpus cavernosum of a hypogonadic rabbit seen three weeks after castration demonstrating moderate vascular spaces (V) within the erectile tissue. Note also the increased quantity of collagenous fibers intermingled with smooth muscle cells within the trabeculae compared to the normogonadic rabbit. TA = Tunica albuginea; Stain = Masson's Trichrome; Magnification = X100.

Figure 11B: Structure of the penile corpus spongiosum (CS) of a hypogonadic rabbit seen three weeks after castration demonstrating moderate vascular spaces within the erectile tissue. sTA = Spongiosal tunica albuginea; U = Penile urethra; Stain = Masson's Trichrome; Magnification = X40.

Figure 11C: Structure of the penile corpus cavernosum of a hypogonadic rabbit seen six weeks after castration demonstrating moderate collapse of vascular spaces (V) within the erectile tissue compared to a normogonadic rabbit; Stain = Masson's Trichrome; Magnification = X100.

Figure 11D: Structure of the penile corpus spongiosum of a hypogonadic rabbit seen six weeks after castration. There was relative collapse of spongiosal vascular spaces with notable fibrosis fusing with the spongiosal tunica albuginea (arrows). U = Penile urethra; Stain = Masson's Trichrome; Magnification = X40.

Figure 11E: Structure of the penile corpus cavernosum of a hypogonadic rabbit seen nine weeks after castration demonstrating marked vascular spaces (V) within the erectile tissue attended by significant cavernosal fibrosis. Notably, numerous red blood cells (red spots) leaked into the cavernosal trabecular tissues, signifying loss of endothelial integrity. Stain = Masson's Trichrome; Magnification = X100.

Figure 11F: Structure of the penile corpus spongiosum of a hypogonadic rabbit seen nine weeks after castration. Notice the marked collapse of vascular spaces within the corpus spongiosum, replaced by spongiosal fibrosis. U = Penile urethra; CS = Corpus spongiosum; Stain = Masson's Trichrome; Magnification = X40.

Figure 11A-F: Morphology of cavernosal and spongiosal vascular spaces

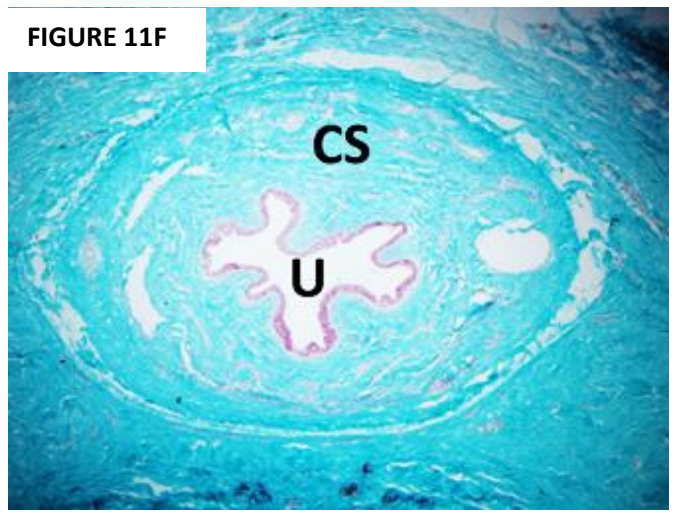
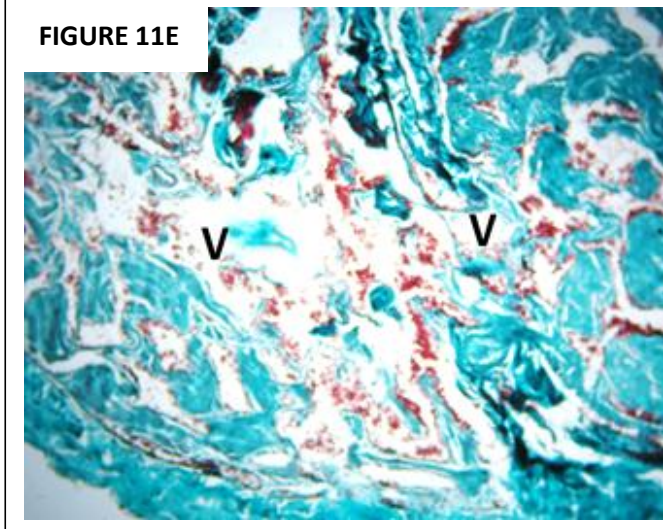
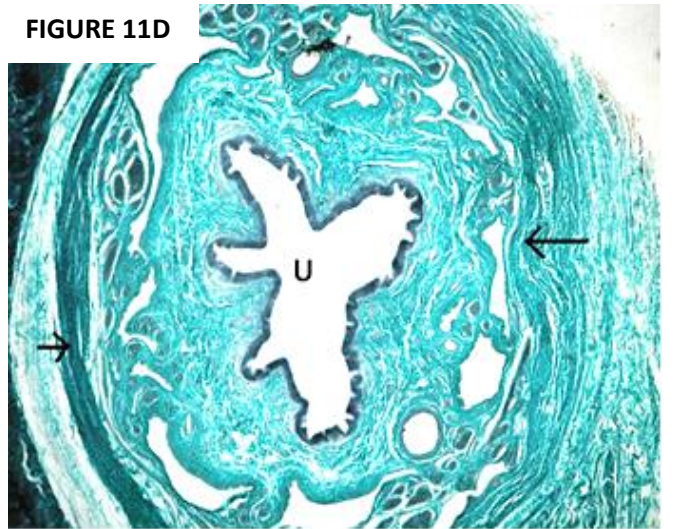
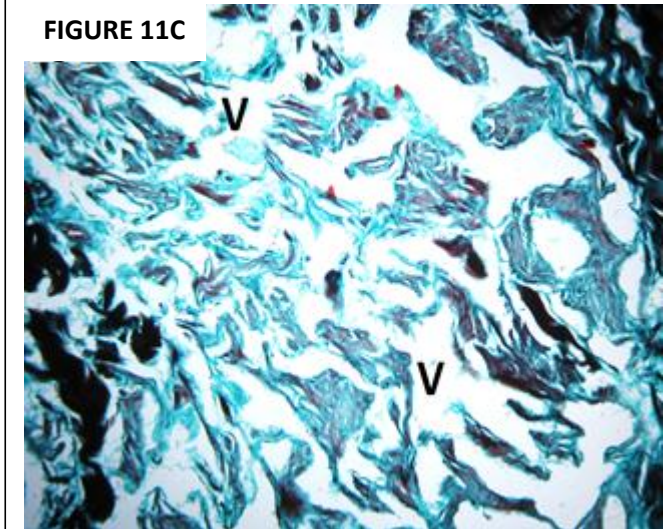
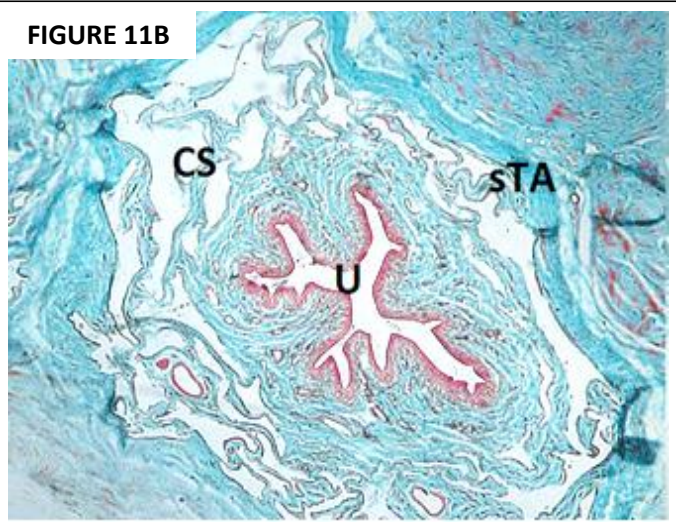
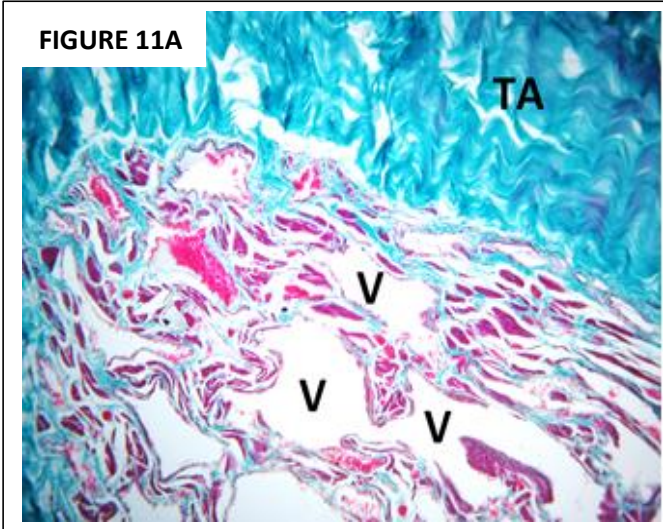


Figure 12A-D: Morphology of cavernosal artery and subtunical veins

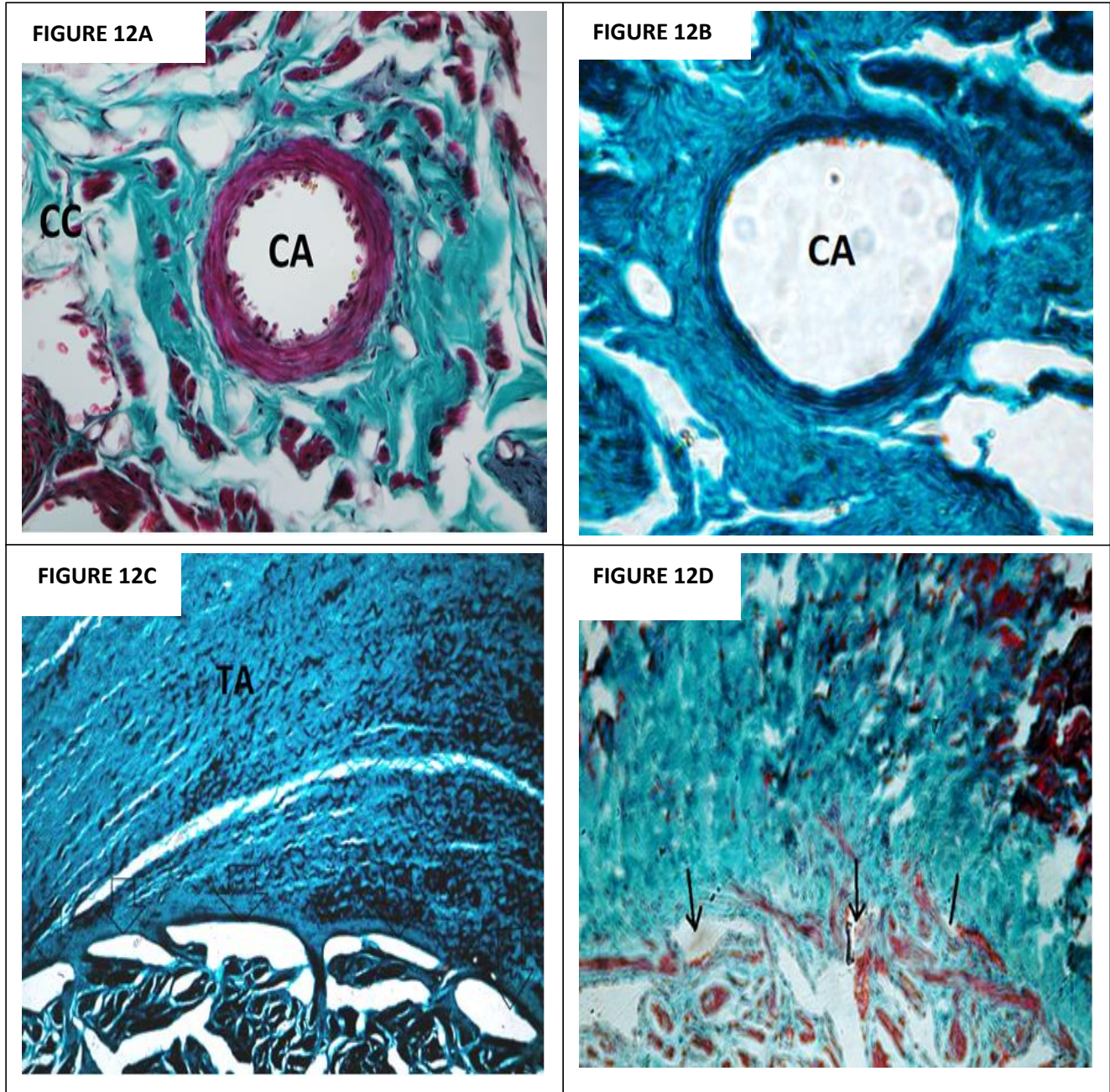
Figure 12A: Structure of the cavernosal artery (CA) of a normogonadic rabbit penis. The artery is well developed with a prominent muscular media. CC = Corpus cavernosum; Stain = Masson's Trichrome; Magnification = X40.

Figure 12B: Structure of the cavernosal artery (CA) of a hypogonadic rabbit penis seen nine weeks after castration. The artery was less muscular, surrounded by marked cavernosal fibrosis. CC = Corpus cavernosum; Stain = Masson's Trichrome; Magnification = X40.

Figure 12C: Structure of the subtunical zone of the penile corpus cavernosum of a normogonadic rabbit. Notice the large and well developed subtunical vascular channels (arrowed); Stain = Masson's Trichrome; Magnification = X100.

Figure 12D: Structure of the subtunical zone of the penile corpus cavernosum of a hypogonadic rabbit nine weeks after castration. There was marked collapse of subtunical vascular channels (arrowed). Stain = Masson's Trichrome; Magnification = X100.

Figure 12A-D: Morphology of Cavernosal Artery and Subtunica Veins



3.6 Comparative Morphometric Analysis of the penile erectile tissue components

The penile erectile tissues consisted of trabeculae and vascular spaces. The trabeculae contained smooth muscles, collagen fibers and elastic fibers. The smooth muscles and collagen fibers were demonstrated using Masson's trichrome stain, while elastic fibers were demonstrated using Weigert's resorcin-fuchsin. Using the slides stained by Masson's trichrome, the vascular spaces occupied about 52.9% of the cavernosal erectile tissue in normogonadic rabbits. In the hypogonadic rabbits, the volumetric densities of the cavernosal vascular spaces were 44.7%, 35.1% and 14.4% after 3 weeks, 6 weeks and 9 weeks respectively (Figure 13A).

Of the cavernosal trabecular tissue, smooth muscle was the most abundant component of the trabeculae in normogonadic rabbits as determined by both direct observation and stereological techniques, constituting about 64.6 % of the penile trabecular. This was closely followed by collagen (35.2%) and elastic fibers (19.4%). In hypogonadic rabbits, however, collagenous fibers were the most abundant component of the trabecular. Their volume density increased proportional to the period of hypogonadism, maximal at 9 weeks after castration. Both smooth muscle and elastic fiber quantity reduced as the period of hypogonadism increased, and the changes were statistically significant ($P < 0.05$). The volumetric densities (V_v) of the various tissue components of the cavernosal erectile tissues of different rabbit groups were as illustrated in figure 13B.

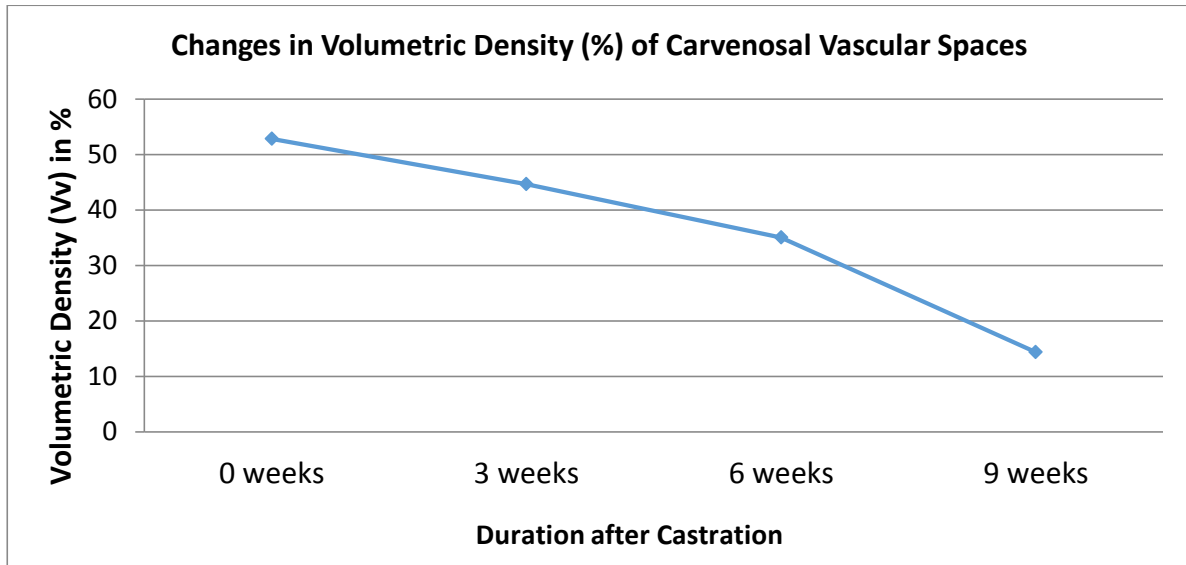


Figure 13A: Volumetric Density of Cavernosal vascular spaces in different rabbit categories. Notice the decline in the proportion of vascular spaces with increasing duration of hypogonadism.

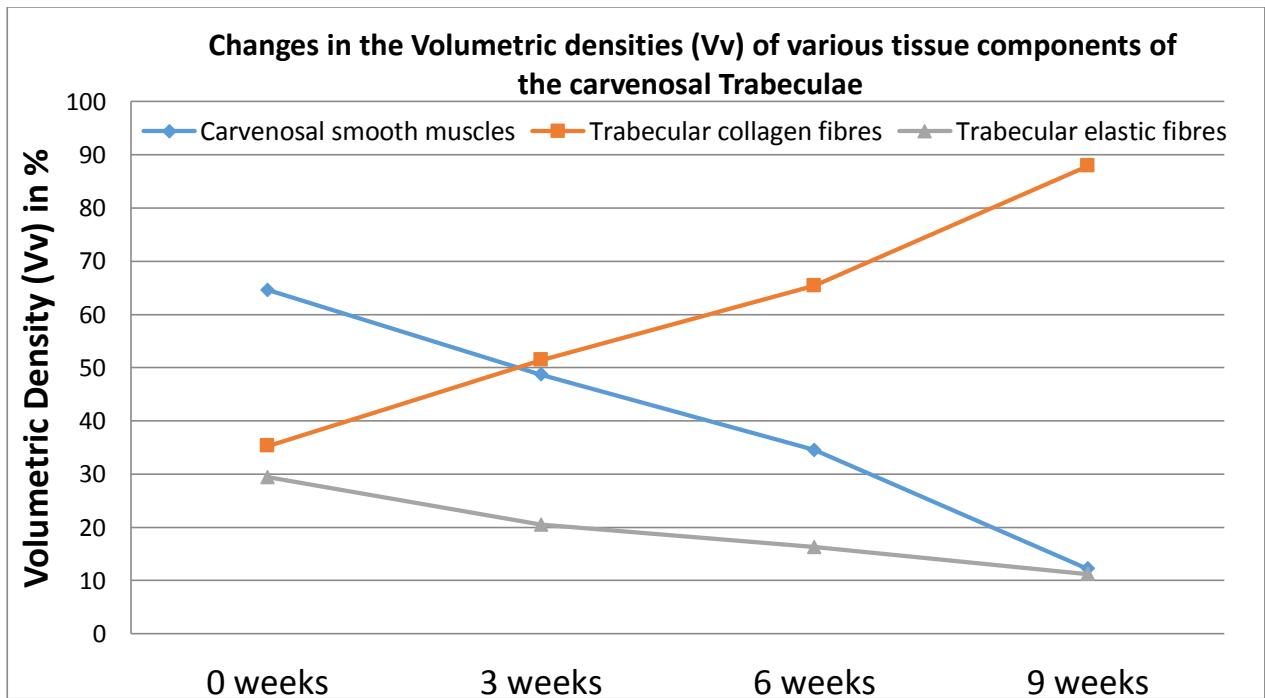


Figure 13B: Volumetric Density (Vv) of various tissue components of the cavernosal trabeculae in different rabbit categories. Notice the progressive decrease in smooth muscle and elastic fibers, and increase in collagenous fibers.

CHAPTER 4: DISCUSSION

Observations of the present study have shown that castration induces diminutive changes in various components of the penile structure, proportional to the duration of exposure to gonadal androgen hormone deprivation. These changes include progressive decrease in penile length, reduction in the quantity of penile smooth muscle cells, marked fibrosis of the erectile masses, disintegration of the tunica albuginea, collapsing of vascular spaces, reduction in elastic fiber content and accumulation of fat cells.

4.1 Shortening of the Penile Length

The present study has shown that bilateral orchiectomy causes a reduction in non-erect penile length. The reduction in penile length is proportional to the duration of hypogonadic exposure. To the best of our knowledge, there are no published reports that have taken the penile length measurements of the non-erect rabbit penis. Previous studies focused mainly on the erect penile length (Haliloglu et al., 2007; McCullough, 2008; Park et al., 2011). Irrespective of the methodology however, all are concordant that the penile length reduces in hypogonadal states. This length reduction may be attributed to many factors such as penile erectile tissue fibrosis, reduction in trabecular smooth muscle density and vascular collapse.

Concordant with current observations, subnormal penile sizes have been reported in androgenic hormone deficiency occurring during embryonic development (Bin-Abbas et al., 1999; Traish and Guay, 2006). The occurrence of congenital micropenis has been shown to depend largely on a critical period of genital development called the “masculinization programming window (MPW)”, which varies in different animals (Welsh et al., 2008). The maximal growth potential of the penis

is in fact predetermined during this period when the level of androgenic activity is high. However, even though androgens are important during this period in programming the penile size, a normal postnatal androgenic action is vital for the penis to achieve this size (Welsh et al., 2008; Macleod et al., 2010). Interestingly, administration of dihydrotestosterone promotes phallic growth in infants and children with micropallus due to 5 α -reductase deficiency (Charmandari et al., 2001).

Current findings and reports from previous studies therefore highlight that penile development occurs in two critical phases, both of which are androgen dependent. Phase I involves penile formation and it occurs early in fetal life (Welsh et al., 2010). It is considered the most important phase since it influences the second phase, and that deficits in androgenic action during this period (MPW) may not be fully repaired by androgen therapy later in life (Bin-Abbas et al., 1999; Welsh et al., 2010). Phase II involves penile growth, and offers a wider time window for androgenic activity until completion of puberty. Current observation also shows that penile size can reduce even after normal development in the setting of gonadal androgen deficiency. This has also been reported in humans suffering from androgen insensitivity (Hlaskova et al., 2009). Normal postnatal androgen levels are therefore vital in maintaining the normal penile size.

4.2 Penile Smooth Muscle Cells

We demonstrated that castration causes significant reduction of the penile trabecular smooth muscles cells. This reduction due to hypogonadism is consistent with other studies (Traish et al., 2003; Traish et al., 2005; Haliloglu et al., 2007; Shafika et al., 2010). The loss of trabecular smooth muscle resulting from androgen deprivation may be attributed to atrophy, an increase in programmed cell death, connective tissue deposition, and adipocyte differentiation from precursor

cells (Shabsigh, 1997; Traish et al, 2005). Accordingly, ultrastructural studies of tissue from castrated animals and hypogonadic men have documented disorganization of smooth muscle cells, with large cytoplasmic vacuoles and decreased myofilament content (Persson et al, 1989; Mersdorf et al, 1991; Traish and Kim, 2005; Traish and Guay, 2006; Traish et al, 2007).

Another possibility explaining reduction of penile smooth muscle is the dedifferentiation of these cells into other phenotypes. In several experimental systems, smooth muscles have been shown to undergo dedifferentiation into other phenotypes (Chamley et al., 1974; Slomp et al., 1997; Johnson et al, 2001; Lehti et al., 2009). Interestingly, inhibition of 5 α -reductase activity induces stromal remodeling and smooth muscle dedifferentiation in the prostate (Corradi et al., 2004). Because 5 α -reductase converts testosterone to dihydrotestosterone (DHT), these data suggest that DHT deficiency may promote smooth muscle dedifferentiation. However, there is no data in the literature on the dedifferentiation of the trabecular smooth muscle in the corpus cavernosum.

Ischemic injury secondary to penile vascular atherosclerosis may be another explanation to the reduction in penile smooth muscle (Wespes et al., 2002; El-Sakka and Yassin, 2010). A direct correlation between penile oxygen tensions has been demonstrated with the percentage of smooth muscle fibers (Sattar et al, 1995; Wespes et al., 2002). There is evidence that androgen deficiency is associated with atherosclerosis, which is a known cause of ischemia (Hak et al., 2002). Studies in humans suggest that androgen deficiency is associated with increased triglycerides, total cholesterol, and low-density lipoprotein cholesterol (Bobjer et al., 2012). Accordingly, androgen deprivation adversely affect lipid profiles (Lateef et al., 2013), but androgen treatment improves lipid profiles and are therefore critical in the prevention and progression of atherosclerosis (Medras and Jankowska, 2001; Schleich and Legros, 2004).

Smooth muscles are considered the cellular targets for direct androgenic effects in penile erectile tissues (Mirone et al., 2009). These cells are vital during erection because they cause dilation of the resistance arterial bed of the penis, providing increased blood flow and pressure to the corpora (Anderson and Wagner, 1995; Lue and Dahiya, 1997). Adequate arterial inflow and trapping of blood within the cavernosal bodies (veno-occlusion) is critical for the development of increasing pressure and volume expansion (Traish and Kim, 2005). Relaxation of penile smooth muscles is necessary for the veno-occlusion of the subtunical veins to occur (Wespes et al, 1997; Rogers et al., 2003; El-Sakka and Yassin, 2010). This veno-occlusive mechanism reduces blood flow from the corpus cavernosum hence contribution to erection (Wespes et al., 1997; Udelson et al., 2000; Kovanecz et al, 2006).

The ability to achieve normal penile corporal veno-occlusion during erection is partially determined by the percentage of corporal smooth muscle content (Wespes et al, 1997). Factors that decrease the content or function of the corporal smooth muscle may therefore predispose to the development of corporal veno-occlusive dysfunction, a known mechanism that underlies erectile dysfunction (Udelson et al., 2000; Eardley, 2002; Rogers et al., 2003; Tsao et al., 2004; Dean and Lue, 2005). Accordingly, severity of symptoms and clinical findings in men with erectile dysfunction has been correlated with reduced tissue content of corporal smooth muscle (Jevtich et al., 1990; Wespes et al, 1997; Nehra et al., 1998). Reduction of smooth muscles observed in the present studies would therefore partly confirm a structural basis of erectile dysfunction in hypogonadism. In humans, the corpora cavernosa in the young are composed of up to 52% smooth muscles and in elderly about 19% to 36% (Wespes et al, 1997).

4.3 Penile collagenous connective tissue

The penile fibrous skeleton consisted of the thick lamellated tunica albuginea and its fibrous columns extending into the erectile tissue, constituting penile trabecular system. The tunica albuginea was observed to be composed of collagenous fibers arranged in undulating thick lamellae, similar to reports from other studies (Hsu et al., 1994a; Brock et al., 1997). The tunica acts as a fibrous frame, with its columns penetrating into various depths within the corpus cavernosum; it prevents overstretching or compression of the vascular and nervous structures, which are under increasing intracavernosal pressure during erection (El-Sakka and Yassin, 2010). Consistent with previous reports, the tunica was a bi-layered structure, having an inner circular and outer longitudinal components (Hsu et al., 1994a; Brock et al., 1997; Hsu et al., 2004). The multilayered nature of the tunica causes sliding hence allowing for flexibility (El-Sakka and Yassin, 2010).

The present study demonstrated that castration causes gradual fibrosis of the penile erectile tissues, with concomitant reduction in trabecular smooth muscle. Such increased deposition of the extracellular matrix have also been reported in other studies involving androgen hormone deficiency (Traish et al, 2003; Traish and Kim, 2005; Haliloglu et al., 2007; Traish, 2009; Shafika et al., 2010) and in aging studies (Ferrini et al., 2001; Shen et al., 2003; Davila et al., 2004; Nolzco et al., 2008; Iacono et al., 2012). Penile erectile tissue fibrosis is due in some cases to the appearance and accumulation of myofibroblasts, or in other cases the switch to a more synthetic phenotype of the original cell components such as fibroblasts (Gonzalez-Cadavid, 2009). The actual mechanisms underlying penile fibrosis in hypogonadism may be complex and via multiple pathways.

To begin with, as already indicated, androgen deficiency is associated with atherosclerosis of blood vessels (Hak et al., 2002; El-Sakka and Yassin, 2010). This is known to cause narrowing of blood vessels leading to reduced blood flow hence ischemia due to low oxygen tension. The relationship of oxygen tension and cavernosal fibrosis has been demonstrated in previous studies (Moreland et al, 1995; Moreland, 1998). There is a likelihood of hypoxia induced overexpression of Transforming Growth Factor – Beta 1 (TGF- β 1) [Moreland, 1998]. TGF- β 1 is pleiotropic cytokine that has been shown to increase cavernosal collagen synthesis in vitro. Under ischemic conditions, it induces its own mRNA, leading to a further increase in TGF- β 1 synthesis that reinforces the development of severe fibrosis (Moreland, 1998). This mechanism may underlie the severe fibrosis observed in the current study after nine weeks of gonadal androgen deprivation. Measurements of differential mRNA expression for various growth factors in young and aging rat penile tissues has also demonstrated that TGF- β 1 is higher in older rats compared with young rats (Dahiya et al, 1999). This also seems to confirm the role of this cytokine in penile fibrosis.

Another possible cause of penile fibrosis after castration can be linked to hypogonadism-induced nerve damage. Cavernosal nerve damage has been associated with corporal fibrosis and loss of cavernosal smooth muscles (Leungwattanakij et al, 2003; Ferrini et al, 2009). An experimental study also demonstrated that protein expression of collagen type I and type III was significantly higher in neurotomy animals compared with a control group (Diegelmann, 1997). Androgens are known to modulate the structure and function of pelvic, cavernosal and dorsal nerves (Meusburger and Keast, 2001; Matsumoto, 2001; Rogers et al., 2003; Armagan et al., 2007). This observation is consistent with those made by Baba et al (2000) in which castration reduced NADPH staining in the cavernosal and dorsal nerves and testosterone treatment restored these nerve fibers to control levels. Androgens promote the expression of vascular endothelial growth factor (VEGF)

[Haggestrom et al, 1999], a known neurotropic factor (Pereira et al., 2011; Pan et al., 2013). It is therefore possible that in the current study there was down-regulation of cavernosal VEGF synthesis in the castrated animals due to androgen deficiency.

Cavernosal nerve damage has also been implicated in penile fibrosis and penile length shortening associated with erectile dysfunctions after prostatectomy procedures (Savoie et al., 2003; User et al., 2003; McCullough, 2008; Yu et al., 2010; Vasconcelos et al., 2012). Because erectile dysfunction is significantly more common in men who undergo non-nerve-sparing prostatectomy than in men who undergo nerve-sparing prostatectomy, a neurogenic cause is recognized to be a main etiology of post-prostatectomy erectile dysfunction (Gralnek et al, 2000).

Other than the ischemic injury and cavernosal nerve damage mechanisms already described, it is still possible that penile tissue fibrosis can be caused by other mechanisms, such as by affecting matrix metalloproteinase (MMP) synthesis and/or activity (Edwall et al., 2007). Some authors have also argued that androgens regulate the growth of smooth muscle and protein synthesis of the connective tissue of the corpora cavernosa, and a decrease in their production could therefore give rise to the switch from elastic fibers to collagen fibers, forming another basis of cavernosal fibrosis (Traish and Guay, 2006; Iacono et al., 2012).

The connective tissue content of the corpora cavernosa relative to the smooth muscle, assessed histologically with specific staining, is defined as the connective tissue to smooth muscle ratio (Traish, 2009). In the present study this ratio was markedly increased in the castrated rabbits, evidenced by the tissue fibrosis seen and the stereological data. This excessive deposition of collagen and extra-cellular matrix with a compensatory decline in trabecular smooth muscle content may alter penile fibroelastic properties (Persson et al, 1989; Mersdorf et al, 1991). Such

changes in tissue architecture has been shown to be associated with reduction in intracavernosal pressure during erection (Traish et al, 2003; Palese et al., 2003; Suzuki et al., 2007), most probably because fibrosis reduces penile compliance and blood flow (Mersdorf et al, 1991; Adams et al., 1997). Erectile tissue fibrosis could therefore form part of the structural basis of erectile dysfunction in hypogonadism.

In the present study we also demonstrated that castration causes progressive deterioration in the tunical integrity. Specifically, it causes disintegration of the collagenous fibers of the tunical albuginea, and accumulation of fat cells. This was evidenced by the progressive loss of the lamellar arrangement of collagenous fibers. To the best of our knowledge, disintegration of the tunica albuginea and intratunical fat cell accumulation following castration is hitherto undescribed. Such irregular arrangement of the tunical collagen fibers may cause noncompliance. The affected area of the tunica albuginea does not expand upon erection and therefore may cause tethering and curvature of the penis (Brock et al, 1997; El-Sakka et al, 1998).

4.4 Penile elastic fiber profile

The present study showed that the normal rabbit penis is a fibroelastic organ with prominent elastic fibers in the erectile tissues. The abundance of elastic fibers have also been noted in other studies of the normal penis (Hsu et al., 1994b; Sattar et al., 1994; Pinheiro et al., 2000; Shen et al., 2003; Babinski et al., 2005; Andrade et al., 2012). Elastic fibers are composite structures composed of a cross-linked elastin core and an outer layer of fibrillin microfibrils (Kielty et al., 2002). These two components perform distinct roles; elastin stores energy and drives passive recoil, whilst fibrillin microfibrils mediate cell signaling, elastogenesis and potentially act to reinforce the elastic fiber.

Generally, the elastic fiber system is characterized by great extension qualities and elastic recoil (Cotta-Pereira et al., 1976). Indeed, the ability of elastic tissues to deform under physiological forces and to subsequently release stored energy to drive passive recoil is vital to the function of many dynamic tissues (Gosline et al., 2002; Sherratt, 2009). The fiber locations and arrangement are related to their different functionality which may reflect local tissue mechanical properties (Cotta-Pereira et al., 1976). Elastic fibers, together with collagen fibers, are therefore important penile constituents that maintain the penile structure during erection, and allow adequate resistance during the return to the non-erect state (Hsu et al., 1994b; Sattar et al., 1994; Bastos et al., 2004).

In the penile trabeculae, elastic fibers form a meshwork in different directions than those of collagen (Pinheiro et al., 2000). They can stretch to 150% of their normal length (Kielty et al., 2002), and are therefore important in erectile tissue erection (Bastos et al., 2004). Accordingly, reduction of elastic fibers in hypogonadic states, as seen in the current study, could be a major factor influencing penile hemodynamic functions. Similar decrement in elastic fiber content has also been reported by other studies (Sattar et al., 1994; Shen et al., 2003). Our study observed a direct relationship between the reduction in elastic fiber content and duration of hypogonadism.

The molecular mechanisms of reduction in elastic fibers are not well explored. However, several studies have suggested that androgens modulate the extracellular matrix through expression of growth factors (Natoli et al., 2005). Interestingly, decrease in penile elastic fibers has also been shown in impotent patients (Sattar et al., 1994; Iacono et al., 1994) and during aging (Sherratt, 2009). Such reduction in elastic fibers alters tissue fibroelastic properties, compromising the penile tissue compliance, and this may result in erectile dysfunction (Jevtich, 1990; Nehra et al., 1998). Reduction in trabecular elastic fiber content therefore may underlie erectile dysfunctions.

Elastic fibers of the tunica albuginea were however scarce in the normogonadic rabbits, and nearly absent in the castrated rabbits. This concurs with a previous study in rats after castration or administration of finasteride (Shen et al., 2003). Ultrastructural findings also confirm these findings, and further show that in normal tunica albuginea, these fibers form an irregular lattice network onto which the collagen fibrils lie (El-Sakka and Yassin, 2010). Together with collagen fibers, they are key in maintaining the compliant nature of the tunica albuginea. They permit the increase in girth and length during tumescence while providing adequate resilience to return rapidly to the flaccid state with detumescence (Hsu et al, 1994b). Changes in elastic fibers can provoke mechanical alterations of the penis, which reduce its elasticity and compliance (El-Sakka and Yassin, 2010). They may also impair the veno-occlusive function of the tunica albuginea (Gentile et al, 1996; Akkus et al, 1997).

4.5 Fat Cell Accumulation

Another striking observation made in this study was the accumulation of fat cells in the subtunical and intratunical zones of the corpus cavernosum. Although subtunical adipocyte accumulation has also been reported in other studies (Moon et al, 2004; Traish et al., 2005; Goyal et al., 2005; Kovanecz et al, 2006), intratunical accumulation is hitherto undescribed. Earlier studies observed the subtunical adiposis after castration (Traish et al., 2005), administration of estrogen hormone (Goyal et al., 2005), administration of endocrine disrupters (Moon et al, 2001; Moon et al, 2004) and in diabetic animals (Traish and Kim 2005; Kovanecz et al, 2006).

Study reports show that androgenic hormones promote differentiation of mesenchymal pluripotent cells into smooth muscle and inhibit their differentiation into adipocytes (Bhasin et al., 2003; Singh

et al., 2003; Singh et al., 2006). On this note, we suggest that castration, hence gonadal hormone deficiency, would favor accumulation of adipocytes in the corpus cavernosum and decrease smooth muscle content, as observed in the present study. It is possible that pluripotent stem cells are present in the corpus cavernosum and that these cells respond to androgen deprivation by differentiation to an adipogenic lineage (Traish et al., 2005). Furthermore, androgen deficiency has been shown to cause a disturbance in lipid profiles, with increase in adiposity (Mauras et al., 1998; Medras and Jankowska, 2001; Schleich and Legros, 2004; Bobjer et al., 2012). Such adiposity, we hypothesize, may include abnormal fat deposition in the penile tissues.

The veno-occlusive mechanism of the penile erection depends partly on the integrity of the subtunical veins that are compressed to impede blood flow from the erectile tissues (Udelson et al., 2000; Traish and Kim, 2005). It is plausible that the presence of fat cell accumulation in the subtunical region of the corpus cavernosum impairs the engagement of this vital mechanism, in addition to causing venous leakage (Traish et al., 2005). As already mentioned, a compromised veno-occlusive mechanism is a known pathway to erectile dysfunction (Udelson et al., 2000; Eardley, 2002; Tsao et al., 2004; Dean and Lue, 2005), and it has been described even in the non-responders to medical management of erectile dysfunctions (Rogers et al., 2003).

4.6 Penile vascular system

The penile vascular system of focus in this study consists of the cavernosal arteries, erectile tissue vascular spaces/sinusoid and the subtunical veins. Castration induced cavernosal arterial fibrosis, vascular leakage and collapse of subtunical veins and vascular spaces. These findings are consistent with the general penile erectile tissue fibrosis observed in this (figures 13A-D) and other

hypogonadic models (Traish et al, 2003; Traish and Kim, 2005; Traish, 2009). The cavernosal artery demonstrated progressive fibrosis in hypogonadic rabbits in the current study. Previous studies have paid little attention to the structure of the cavernosal artery in hypogonadism. However, an identical loss of smooth muscle and increased fibrosis has been observed in the penile dorsal artery and aorta in diabetic rat models (Kovanecz et al., 2009). Penile fibrosis has also been shown to occur with increase in age (Shen et al., 2003, Iacono et al., 2012) and cavernosal nerve damage (Leungwattanakij et al., 2003; Ferrini et al., 2009).

Diffuse and progressive intra-corporal fibrosis similar to the one observed in hypogonadic rabbits in the present study is known to be responsible for causing vasculogenic erectile dysfunction associated with aging, smoking, diabetes, hypertension, and post-radical prostatectomy (Gonzalez-Cadavid, 2009). Such changes may be caused by increased oxidative stress and/or other profibrotic factors that stimulate smooth muscle apoptosis and collagen deposition within these vessels (Ferrini et al., 2001). The molecular links between hypogonadism and collapse of vascular spaces/fibrosis could be multiple.

One possible mechanism is ischemic injury which leads to fibrosis. Androgen deficiency has been associated with the progression of atherosclerosis, production of pro-inflammatory cytokines, increased arterial thickness, increased levels of glucose, total cholesterol, and low-density lipoprotein (Hak et al., 2002; Miller et al., 2004; Francomano et al., 2010). Atherosclerotic changes are known to cause narrowing of blood vessels hence reduced blood flow. The initial insult to any of the penile tissues generally results in the release of profibrotic factors, mainly transforming growth factor beta 1 (TGF- β), plasminogen activator inhibitor 1, and reactive oxygen species leading to oxidative stress (Gonzalez-Cadavid, 2009). These are associated with fibrosis.

Androgens exert a number of beneficial effects on both cardiovascular and penile tissues. Endothelial cells and smooth-muscle cells are the main cellular targets for androgenic effects in both tissues (Mirone et al., 2009). For this reason, hypogonadism is a condition associated with endothelial dysfunction (Akishita et al., 2007; Foresta et al., 2008). A study by Lu et al (2007) demonstrated that androgen deprivation causes damage to the endothelium as determined by electron microscopy, and administration of testosterone into the deprived animals partially restored the endothelial structural integrity. The restoration or remodeling of endothelial injury depends, in part, on a pool of circulating progenitor cells, which are generally depleted in androgen deficiency (Foresta et al., 2006; Foresta et al., 2008; Traish, 2009).

Physiological concentrations of testosterone (and DHT) have been shown to increase endothelial synthesis of nitric oxide, a key vasodilator molecule in the penile erectile tissues (Miller and Mulvagh, 2007; Goglia et al., 2010; Campelo et al., 2012). Experimental data also demonstrate that testosterone induces relaxation of many vascular beds (Yue et al., 1995; English et al., 2002; Campelo et al., 2012). Therefore, within the penile tissues from castrated rabbits studied here, it is plausible that vasoconstrictive tone may have overridden the vasodilatory mechanisms due to androgen deficiency. Accordingly, men with low free testosterone have impaired erectile tissue vasodilation, hence they present with erectile dysfunction (Aversa et al., 2000; Aversa et al., 2003).

LIMITATIONS OF THE STUDY

The following were limitations encountered in this study:

1. Castration is a surgical procedure that causes tissue injury. For this reason some of the penile changes may have been due to the reactive process of the tissues to injury. This was delimited by the fact that we used the prescrotal approach which does not tamper with the penis. Also based on the fact that the changes observed in the study were proportional to the duration of gonadal hormone deficiency, they are most likely have been due to the androgen deficiency rather than surgical trauma.
2. We were unable to determine whether the reduction in number of smooth muscles was due to apoptosis or atrophy or both.

CONCLUSION

In addition to gross morphological penile length reduction, castration induces diminutive changes in all tissue components of the penile structure namely the smooth muscle, connective tissue and vascular sinusoids. These changes included reduction in the non-erect penile length, trabecular smooth muscle cells and elastic fibers, increase in collagen with erectile tissue fibrosis, disruption of the lamella arrangement of collagen fibers of the tunica albuginea and disintegration of the intercavernosal septum, cavernosal artery fibrosis, loss of endothelial integrity, narrowing or collapse of subtunical vessels and fat cell accumulation. These anatomical changes may impair penile physiology hence forming anatomical basis of erectile dysfunctions in hypogonadism.

SUGGESTIONS FOR FURTHER STUDIES

1. Immunohistochemical studies to determine the actual mechanism that underlie depletion of smooth muscle cells.
2. Electron microscopic studies to determine the pattern and extent of endothelial damage.
3. Induction of hypogonadism by pharmacological castration to minimize the possible impact of surgical castration on the penile structure.

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APPENDIX

Data Sheet 1: Penile length measurements

Rabbit group	S.No	Penile Length			
		At start	After 3 weeks	After 6 weeks	After 9 weeks
Intervention group	C1				
	C2				
	C3				
	C4				
	C5				
	C6				
	C7				
	C8				
	C9				
Non-intervention group	N1				
	N2				
	N3				
	N4				
	N5				
	N6				

KEY: C = Castrated; N = Normo-gonadic

Data Sheet 2: Volumetric densities

Rabbit serial number:.....

Section	1	2	3	4	5	6	7	8	9	10	Average
Elastic fibers											
Collagen fibers											
Smooth muscles											
Vascular spaces											
Total											